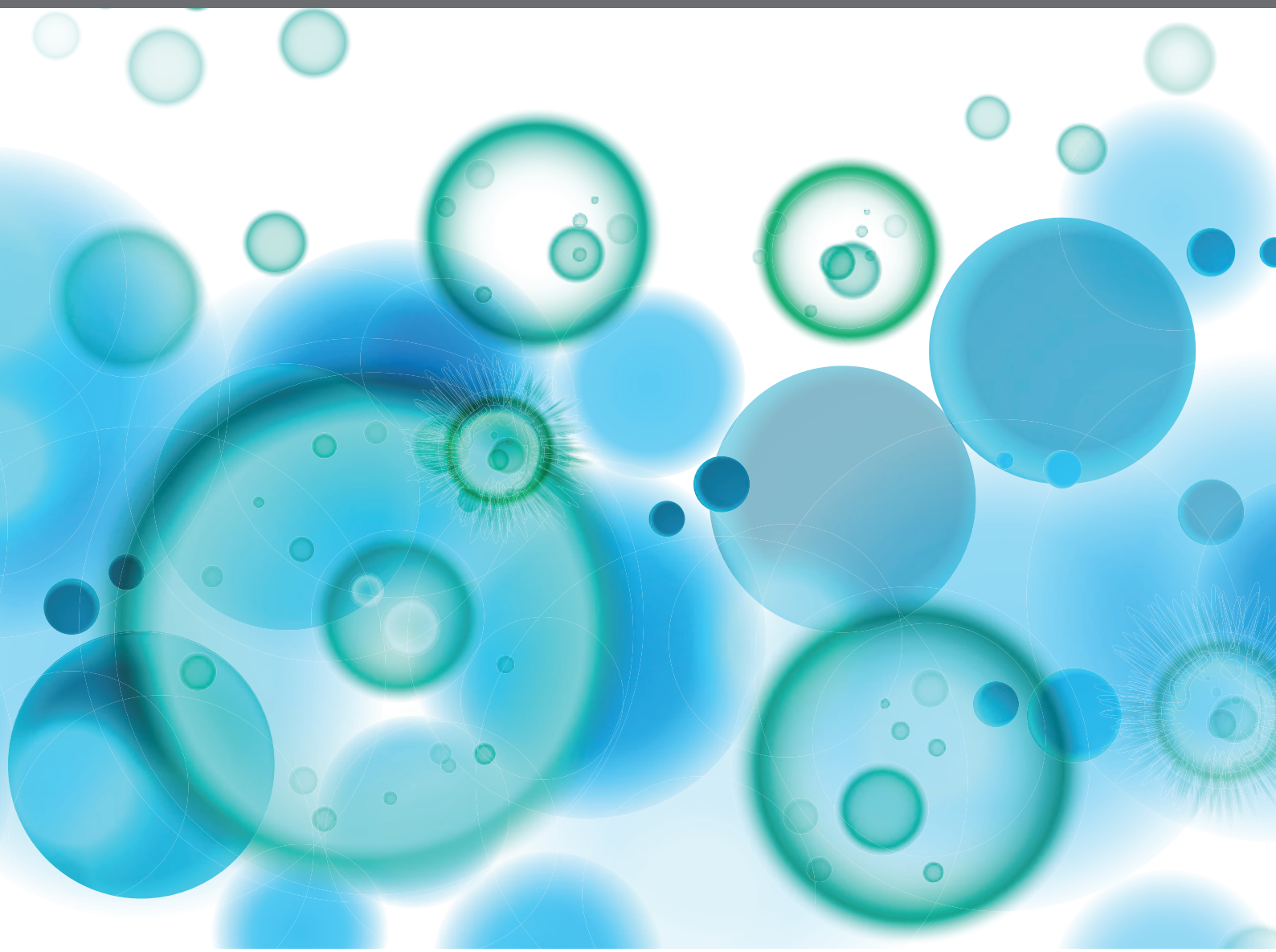
ROLE OF LIPIDS IN THE DYNAMICS OF ALLERGIC AIRWAY INFLAMMATION

EDITED BY: Nestor González Roldán, Otto Holst, Johannes Huebner and Katarzyna Anna Duda

PUBLISHED IN: Frontiers in Immunology



frontiers Research Topics

frontiers

ISSN 1664-8714
ISBN 978-2-88966-315-6
DOI 10.3389/978-2-88966-315-6

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to Quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: researchtopics@frontiersin.org

ROLE OF LIPIDS IN THE DYNAMICS OF ALLERGIC AIRWAY INFLAMMATION

Topic Editors:
Nestor González Roldán, Research Center Borstel (LG), Germany
Otto Holst, Research Center Borstel (LG), Germany
Johannes Huebner, Ludwig Maximilian University of Munich, Germany
Katarzyna Anna Duda, Research Center Borstel (LG), Germany

Citation: Roldán, N. G., Holst, O., Huebner, J., Duda, K. A., eds. (2021). Role of Lipids in the Dynamics of Allergic Airway Inflammation. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88966-315-6

Table of Contents

frontiers in Immunology

EDITORIAL
published: 04 November 2020
doi: 10.3389/fimmu.2020.612297

Editorial: Role of Lipids in the Dynamics of Allergic Airway Inflammation

Nestor González Roldán * *and Katarzyna Anna Duda*

Group of Allergobiochemistry, Priority Research Area Asthma & Allergy, Research Center Borstel, Leibniz Lung Center, Member of the German Center for Lung Research (DZL), Airway Research Center North (ARCN), Borstel, Germany

Keywords: lipid adjuvants, natural killer T cell, asthma, structure-activity relationship, allergic inflammation, allergen-specific immunotherapy

Editorial on the Research Topic

Role of Lipids in the Dynamics of Allergic Airway Inflammation

Allergic airway inflammation is a multifactorial and complex process considered to be an abnormally exacerbated reaction towards otherwise innocuous common environmental factors, such as pollen grains, dust mites, or their molecular components. It involves several cell types of the innate and adaptive immune system, including airway epithelial cells, ILC2, Th2 cells, mast cells, eosinophils, and basophils. The Th2-related and prototypical cytokines IL-4, IL-5, IL-9, and IL-13; and the production of allergen-specific IgE are also part of this type of inflammation. When allergic airway inflammation becomes chronic, it leads to the development of asthma. At an early age, allergic sensitization towards proteins takes place in susceptible individuals. In most cases, allergens are proteins from diverse origins and functions, which under normal circumstances would not be recognized by the host's immune system as harmful or dangerous (antigenic). Thus, over the course of decades, protein-allergens have been the focus of research regarding allergic airway inflammation. But, what makes a protein become an allergen? Despite the significant progress in the identification and molecular characterization of allergens, this key question remains unanswered or can only be partially explained, indicating that there are other factors involved. As highlighted by Platts-Mills (1), allergens do not arrive in the airways alone. Their delivery occurs exclusively in particles, which consist of a complex mixture of chemically different molecules, including lipids. Hence, allergic sensitization is a multifactorial process that is not only regulated by the intrinsic biological activity (i.e. protease activity) of the allergens but also by bioactive lipids. Pollen grains evidence very clearly this concept, as they have a rich lipidome of their own (Dahl). These external lipids are either associated to the allergens as ligands, as a component of the allergenic particles, or are derived from microorganisms present on the allergen source (Lembo-Fazio et al.).

By definition, lipids are hydrophobic or small amphipathic molecules, classified into eight well-defined categories: fatty acids, glycerolipids, glycerophospholipids, sphingolipids, sterol lipids, prenol lipids saccharolipids, and polyketides (2). It has been shown that endogenous lipid mediators are crucial for the control of antigen presentation by dendritic cells in the context of asthma (Debeuf and Lambrecht), and for the reactivity of mast cells during the effector phase of allergic inflammation (Hagemann et al.). There is now a growing body of evidence indicating that not only endogenous, but exogenous lipids (not synthetized by host's cells) present in allergenic particles, can target different cell populations of the immune system (3) (Lee et al.). Some examples

OPEN ACCESS

Edited and reviewed by:
Silvano Sozzani,
Sapienza University of Rome, Italy

***Correspondence:**
Nestor González Roldán
ngonzalez@fz-borstel.de

Specialty section:
This article was submitted to Cytokines and Soluble Mediators in Immunity, a section of the journal Frontiers in Immunology

Received: *30 September 2020*
Accepted: *16 October 2020*
Published: *04 November 2020*

Citation:
González Roldán N and Duda KA (2020) Editorial: Role of Lipids in the Dynamics of Allergic Airway Inflammation. Front. Immunol. 11:612297. doi: 10.3389/fimmu.2020.612297

are the phytoprostanes released from grass pollen were shown to prime dendritic cells towards glycolipid presentation and enhance mast cell degranulation (González Roldán et al.). And a glycolipid isolated from *Aspergillus fumigatus* activated NKT cells, inducing airway hyperresponsiveness in an experimental model of asthma (4). Therefore, it is very likely that lipids are the "missing" additional factor involved in the regulation of what we call the "dynamics of allergic inflammation": the transition from one disease stage to the next one, sensitization-chronification-exacerbation. Following this idea: in susceptible subjects during the early phase of exposure, lipids contained in the allergenic particles (working as an adjuvant) could favor the release of the early IL-4 required for the switch to produce IgE by B-cells, resulting in sensitization (5). Once the individual is sensitized to a particular allergen, the repeated contact with bioactive lipids, could provide a background "pre-activation" status, by influencing cellular processes such as calcium mobilization or by keeping a pro-allergic microenvironment. And finally, once allergic inflammation has become chronic, the encounter with allergenic particles has the potential to provoke an exacerbation on its own, but the additional presence of lipids, either alone or working in a synergistic manner, potentiate the intensity of the response.

A plausible explanation why lipids have remained out of focus of study in the field of allergic inflammation, may reflect the challenges in working with lipids under laboratory conditions. With the exception of lipid mediators, the majority of lipids are water-insoluble. Under hydrophilic conditions lipids tend to form micelles or adhere to the walls of tests tubes, becoming not bioavailable. Thus, their hydrophobic properties hinder the analysis of their biological activity in water-based media cultures used in *in vitro* systems. To overcome this issue, researchers often try to solubilize lipids using organic solvents such as DMSO or ethanol, or detergent solutions, that are either toxic for the cells or do not reflect the natural way of delivery to host's cells. In nature, lipids are often bound or associated to diverse lipid transfer proteins, allowing their delivery in a hydrophobic milieu to the target cells (Scheurer and Schülke). The lipid-allergen association can protect allergens from degradation and increase their allergenicity by bringing its own lipid-adjuvant, and thus enhancing the sensitization towards these proteins (Jappe et al.).

Speaking of lipid classes, one has to take into consideration the existence of a high structural/chemical heterogeneity between and inside each class. And subtle structural changes like the targeted addition/removal of functional groups can render lipids either inactive, make them more potent or even induce a shift of the inflammatory response (i.e. Th1 *vs.* Th2). This structure-activity relationship can be exemplified looking at the recognition of glycolipids presented on CD1d by NKT cells. The length of the fatty acid chains and the presence, and location of double bonds affects their affinity for the hydrophobic pockets of CD1d required for loading; and influences the orientation of the carbohydrate part that is recognized by the TCR of NKT cells. As a consequence, these structural cues determine the type of cytokine response by NKT cells (6). However, for other lipid classes than glycolipids, the structure-activity relationship behind the inflammatory response towards an allergic phenotype remains poorly understood. We believe that further detailed structural work, along with the elucidation of receptors, pathways, and cells involved in lipid recognition will set the rational base for the use of lipids as adjuvants for the development of suitable allergen-lipid formulations directed to be used as prophylactic for individuals at risk of developing allergy, or for allergen-specific immunotherapy.

With this Research Topic we aim to call readers' attention towards lipids as important regulators of allergic inflammation, provide an overview of current knowledge, with a focus on structure-activity relationship.

AUTHOR CONTRIBUTIONS

NG and KD wrote the manuscript. All authors contributed to the article and approved the submitted version.

ACKNOWLEDGMENTS

KD and NG would like to thank Prof. Holst and Prof. Hübner for their substantial contribution and support for the successful achievement of this Research Topic.

REFERENCES

1. Woodfolk JA, Commins SP, Schuyler AJ, Erwin EA, Platts-Mills TAE. Allergens, sources, particles, and molecules: Why do we make IgE responses? *Allergol Int* (2015) 64:295–303. doi: 10.1016/j.alit.2015.06.001
2. Fahy E, Cotter D, Sud M, Subramaniam S. Lipid classification, structures and tools. *Biochim Biophys Acta (BBA) - Mol Cell Biol Lipids* (2011) 1811:637–47. doi: 10.1016/j.bbalip.2011.06.009
3. Bublin M. Do lipids influence the allergic sensitization process? *J Allergy Clin Immunol* (2014) 134:521–9.
4. Albacker LA, Chaudhary V, Chang Y-J, Kim HY, Chuang Y-T, Pichavant M, et al. Invariant natural killer T cells recognize a fungal glycosphingolipid that can induce airway hyperreactivity. *Nat Med* (2013) 19:1297–304. doi: 10.1038/nm.3321
5. Yoshimoto T. The Hunt for the Source of Primary Interleukin-4: How We Discovered That Natural Killer T Cells and Basophils Determine T Helper Type 2 Cell Differentiation In Vivo. *Front Immunol* (2018) 9:1–8. doi: 10.3389/fimmu.2018.00716
6. Birkholz AM, Kronenberg M. Antigen specificity of invariant natural killer T-cells. *Biomed J* (2015) 38:470–83. doi: 10.1016/j.bj.2016.01.003

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 19 March 2018
doi: 10.3389/fimmu.2018.00455

Induction of Interleukin-10 Producing Dendritic Cells As a Tool to Suppress Allergen-Specific T Helper 2 Responses

*Stefan Schülke**

Vice President's Research Group 1, Molecular Allergology, Paul-Ehrlich-Institut, Langen, Germany

Dendritic cells (DCs) are gatekeepers of the immune system that control induction and polarization of primary, antigen-specific immune responses. Depending on their maturation/activation status, the molecules expressed on their surface, and the cytokines produced DCs have been shown to either elicit immune responses through activation of effector T cells or induce tolerance through induction of either T cell anergy, regulatory T cells, or production of regulatory cytokines. Among the cytokines produced by tolerogenic DCs, interleukin 10 (IL-10) is a key regulatory cytokine limiting und ultimately terminating excessive T-cell responses to microbial pathogens to prevent chronic inflammation and tissue damage. Because of their important role in preventing autoimmune diseases, transplant rejection, allergic reactions, or in controlling chronic inflammation DCs have become an interesting tool to modulate antigen-specific immune responses. For the treatment of allergic inflammation, the aim is to downregulate allergen-specific T helper 2 (Th2) responses and the associated clinical symptoms [allergen-driven Th2 activation, Th2-driven immunoglobulin E (IgE) production, IgE-mediated mast cell and basophil activation, allergic inflammation]. Here, combining the presentation of allergens by DCs with a pro-tolerogenic, IL-10-producing phenotype is of special interest to modulate allergen-specific immune responses in the treatment of allergic diseases. This review discusses the reported strategies to induce DC-derived IL-10 secretion for the suppression of allergen-specific Th2-responses with a focus on IL-10 treatment, IL-10 transduction, and the usage of both whole bacteria and bacteria-derived components. Interestingly, while IL-10-producing DCs induced either by IL-10 treatment or IL-10 transduction are arrested in an immature/semi-mature state, treatment of DCs with live or killed bacteria as well as isolated bacterial components results in the induction of both anti-inflammatory IL-10 and pro-inflammatory, Th1-promoting IL-12 secretion often paralleled by an enhanced expression of co-stimulatory molecules on the stimulated DCs. By the secretion of DC-derived exosomes or CC-chemokine ligand 18, as well as the expression of inhibitory molecules like cytotoxic T lymphocyte-associated antigen 4, TNF receptor superfamily member 4, Ig-like transcript-22/cluster of differentiation 85, or programmed death-1, IL-10-producing DCs have been repeatedly shown to suppress antigen-specific Th2-responses. Therefore, DC-based vaccination approaches hold great potential to improve the treatment of allergic diseases.

Keywords: dendritic cells, interleukin-10, allergy, T helper 2, dendritic cell vaccine

OPEN ACCESS

Edited by:
Nestor González Roldán, Allergobiochemie, Forschungszentrum Borstel, Germany

Reviewed by:
Angela Bonura, Consiglio Nazionale Delle Ricerche (CNR), Italy
Raymond P. Donnelly, United States Food and Drug Administration, United States

***Correspondence:**
Stefan Schülke
stefan.schuelke@pei.de

Specialty section:
This article was submitted to Cytokines and Soluble Mediators in Immunity, a section of the journal Frontiers in Immunology

Received: *04 January 2018*
Accepted: *20 February 2018*
Published: *19 March 2018*

Citation:
Schülke S (2018) Induction of Interleukin-10 Producing Dendritic Cells As a Tool to Suppress Allergen-Specific T Helper 2 Responses. Front. Immunol. 9:455. doi: 10.3389/fimmu.2018.00455

INTRODUCTION

Dendritic Cells (DCs) Control the Induction of Immune Responses

Our immune system efficiently protects us from most pathogens. However, if the actions of immune cells are misdirected (e.g., against our own cells and tissues in the case of autoimmune diseases or against innocuous environmental antigens in the case of allergies) severe immunopathology can be the consequence. Therefore, the induction of potentially highly destructive immune responses needs to be tightly regulated.

Usually, the prevention of such detrimental immune responses is achieved by controlling against which antigens cells of the adaptive immune system are allowed to react. Here, antigen-presenting cells (APCs) are pivotal in controlling the induction of innate and subsequent adaptive immune responses.

Antigen-presenting cells consist of DCs, macrophages, and B cells (1, 2). They control both the induction and regulation of T-cell immune responses *via* the uptake, processing, and presentation of antigens to antigen-specific T cells (1, 2).

Among the different types of APCs, DCs are of special importance because they are the only APC type able to induce activation, differentiation, and expansion of naive, antigen-specific T cells (3, 4). In contrast to this, macrophages and B cells are only sufficient to reactivate T cells that have already encountered their specific antigen in the past (5).

Dendritic cells are highly specialized APCs strategically located in the skin and the mucosal system (2, 6). They act as sentinel cells that initiate, monitor, and regulate immune responses (1). In their immature form DCs continuously take up and process antigens *via* endocytosis or pinocytosis (7). If this antigen uptake occurs in the context of additional DC-activating signals such as pro-inflamatory cytokines [tumor necrosis factor alpha (TNF-α), interleukin (IL)-1β, or IL-6], prostaglandin hormones (prostaglandin E 2), immune stimulating bacterial and viral components [lipopolysaccharide (LPS), CpG-DNA; Pam_2CysK_4, flagellin, etc.], or cell-contact-dependent signals [e.g., *via* cluster of differentiation (CD)40-ligand] DCs become activated (8). Once activated, DCs start to present the processed antigens in the context of major histocompatibility complex II (MHC II) molecules and express co-stimulatory molecules on their surface (2, 8). *Via* the expression of the chemokine receptor 7 (CCR7, whose ligand is abundantly expressed in lymph nodes) mature DCs also start to migrate to lymph nodes, where DCs encounter antigen-specific naive T cells and initiate their priming (9, 10).

By their actions, DCs link innate and adaptive immune responses by connecting the detection of danger signals with the uptake, processing, and presentation of foreign antigens to control both the induction and polarization of primary antigen-specific $CD4^+$ T-cell responses (11, 12).

Besides their important function in the induction of antigen-specific immune responses, DCs are also key players in maintaining immune homeostasis (13). Uptake and presentation of innocuous foreign- and self-antigens by DCs usually mediates T-cell tolerance (14). In this context, the cytokine IL-10 has been shown to shift DC function toward a tolerogenic rather than an immunogenic phenotype (15).

Dendritic cells may acquire tolerogenic properties either by (1) displaying a semi-mature state and exert tolerogenic function *via* the induction of apoptosis or anergy in the absence of co-stimulatory signals (2, 3, 16) promoting the differentiation of interacting T cells into $CD4^+CD25^+$ regulatory T (Treg) cells, or (3) increasing IL-10 production to expand allergen-specific type 1 regulatory T (Tr1) cells (3, 17, 18). Indeed, the T cell skewing capacity of DCs largely depends on their cytokine pattern and expression of co-stimulatory molecules (19, 20).

Therefore, depending on their maturation/activation status, the molecules expressed on their surface, and their cytokine production DCs have been shown to elicit immune responses through either activation of effector T cells, induction of tolerance through regulatory T cells, or the induction of regulatory cytokines (6).

Because of their important role in the induction of both innate and adaptive immune responses, DCs have become an interesting tool to modulate antigen-specific immune responses (11, 21). In this context, their capacity to induce, modulate, and control T cell responses makes DCs an attractive adjuvant in vaccination settings that have the aim to either enhance inadequate immune responses for the treatment of infectious diseases and cancer or to attenuate exaggerated immune responses in

Abbreviations: AIT, allergen-specific immunotherapy; AHR, airway hyperreactivity; APC, antigen-presenting cell; AR, allergic rhinitis; Art v1, major mugwort allergen *Artemisia vulgaris* allergen 1; B7-DC, programmed cell death 1 ligand 2 (CD273); BAL, bronchoalveolar lavage; BATF3, basic leucine zipper ATF-like 3; BCG, *Bacillus* Calmette–Guérin*;* Bet v 1, major birch pollen *Betula verrucosa* allergen 1; BM-DCs, bone marrow-derived DCs; cAMP, cyclic adenosine monophosphate; CCL-3/18, CC-chemokine ligand 3/18; CCR5/7, CC chemokine receptor 5/7; CD, cluster of differentiation; cDC, conventional DC; CpG-ODN, oligodeoxynucleotides containing CpG motifs; CTLA-4, cytotoxic T lymphocyte-associated antigen 4 (CD152); DC, dendritic cell; Der p 1, major house dust mite allergen *Dermatophagoides pteronyssinus* peptidase 1; DTH, delayed-type hypersensitivity; FcεRI, high affinity IgE receptor Fc epsilon receptor I; Foxp3, forkhead box protein 3; HLA-DR, human leukocyte antigen DR; ICAM-1, intercellular adhesion molecule 1; ICOS, inducible co-stimulator; ICOS-L, inducible co-stimulator ligand; IDO, indoleamine 2,3-dioxygenase; IFN-α1, interferon alpha 1; IFN-γ, interferon gamma; Ig E/G, immunoglobulin E/G; IL, interleukin; IL-1RN, interleukin 1 receptor antagonist; IL-10R, interleukin 10 receptor; iTregs, inducible Tregs; Jak1, Janus kinase 1; LAG-3, lymphocyte-activation gene 3; LZT, low zone tolerance; MAP kinase, mitogen-activated protein kinase; mDC, myeloid DCs; MHC I/II, major histocompatibility complex I/II; MoDC, monocyte-derived DCs; MPLA, monophosphoryl lipid A; mTOR1, mammalian target of rapamycin 1; MyD88, myeloid differentiation primary response 88; OIT, oral immunotherapy; OX40, TNF receptor superfamily member 4 (CD134); OVA, ovalbumin; PAMP, pathogen-associated molecular pattern; PBMC, peripheral blood mononuclear cell; pDC, plasmacytoid DC; PD-1/2, programmed death-1/2; PD-L1/2, programmed death ligand ½; PDE4, phosphodiesterase-4; PRR, pathogen-recognition receptor; rFlaA, recombinant TLR5-ligand flagellin A; rFlaA:Artv1, recombinant fusion protein consisting of flagellin A and Art v 1; rFlaA:Betv1, recombinant fusion protein consisting of flagellin A and Bet v 1; rFlaA:OVA, recombinant fusion protein consisting of flagellin A and OVA; SbsC, surface (S-layer) protein of *Geobacillus stearothermophilus;* SbsC:Bet v 1, recombinant fusion protein consisting of SbsC and Bet v 1; SLIT, sublingual immunotherapy; SOCS-3, suppressor of cytokine signaling 3; STAT 1/5/6, signal transducer and activator of transcription 1/5/6; T-bet, T-box transcription factor TBX21; TGF-β, transforming growth factor beta; Th0/1/2/17, T-helper 0/1/2/17 cell; TLR, toll-like receptor; TRIF, TIR-domain-containing adapter-inducing interferon-β; TNF-α, tumor necrosis factor alpha; Tr1, regulatory T cell type I; Treg, regualory T cell; Tyk2, tyrosine kinase 2.

conditions such as autoimmunity, allergy, transplant rejection, and chronic inflammation (11, 21).

IL-10 Is an Important Cytokine Limiting Excessive Immune Responses

As we have just seen, cytokines produced by DCs play a central role in controlling both the induction and polarization of primary antigen-specific T-cell responses.

Among the cytokines produced by DCs, IL-10 is a key regulatory cytokine limiting and ultimately terminating excessive T-cell responses to microbial pathogens to prevent chronic inflammation and tissue damage (15, 22). IL-10 can both be produced by and has pleiotropic effects on multiple cell types, including DCs, macrophages, B cells, natural killer cells, both Th1- and Th2 cells, $CD4^+CD25^+$ forkhead box protein 3 ($Foxp3^+$) Treg cells, and keratinocytes (1, 23–25).

Interleukin-10, originally identified as an inhibitor of interferon gamma (IFN-γ) and IL-2 synthesis in Th2 cells (26), efficiently inhibits proliferative and cytokine responses in T cells (1) and was shown to mediate both immunological unresponsiveness and the suppression of immune reactions (27). At epithelial interfaces to the environment, including the skin, IL-10 prevents excessive immune responses to foreign antigens (25).

Indeed, a well-documented mechanism by which IL-10-producing DCs suppress allergic Th2-responses is the induction of allergen-specific $CD4^+CD25^+Foxp3^+$ Treg cells (27–29). For example, Pacciani et al. reported that IL-10-producing DC can induce allergen-specific regulatory T cells suppressing proliferation and inflammatory cytokine production from Th2 cells of the same specificity from house dust mite-allergic patients (30). In line with this, Oh and colleagues showed that IL-10-secreting T cells in the airways were able to reduce Th2-type inflammation and airway hyperreactivity (AHR) (31). Therefore, the induction of IL-10- and transforming growth factor beta (TGF-β)-producing regulatory T cells by IL-10-producing DCs is an important mechanism to prevent excessive immune responses (32, 33).

Consequently, IL-10-deficient mice develop increased contact hypersensitivity (34), display spontaneous enterocolitis and other symptoms akin to Crohn's disease (35), and develop exaggerated asthmatic and allergic responses (35).

Interleukin-10 signaling is transmitted through a heterotetrameric interleukin 10 receptor (IL-10R) which consists of two ligand-binding IL-10R alpha chains and two accessory, signal-transducing beta chains all belonging to the interferon receptor family (**Figure 1**) (23). The IL-10R α chain is expressed at high levels on both macrophages and DCs, whereas the IL-10R β chain is ubiquitously and constitutively expressed by all cell types (23).

Mechanistically, IL-10 inhibits the function of APCs, including macrophages and DCs, by downregulating their maturation status and reducing the associated production of pro-inflammatory cytokines (such as IL-1β, IL-6, or TNF-α), while increasing the expression of inhibitory genes (23, 36). These effects of IL-10 are mediated *via* the Janus kinase 1 (JAK1)/Tyk2/STAT3 pathway. It is initiated when IL-10 homodimers bind to the extracellular portion of the IL-10R alpha chain (**Figure 1**). In a first activation step, IL-10 binding triggers the recruitment of Jak1 to the IL-10R alpha chain and its subsequent phosphorylation, while tyrosine kinase 2 (Tyk2) is recruited to and phosphorylated by the IL-10R beta chain (**Figure 1**) (37, 38). Upon their phosphorylation, these kinases phosphorylate the tyrosine motifs Y446 and Y496 located in the intracellular portion of the IL-10R alpha chain (**Figure 1**) (39). The activated IL-10 receptor complex then mediates the phosphorylation of signal transducer and activator of transcription 3 (STAT3) by providing transient anchorage sites for STAT-3 that allow the phosphorylation of STAT3 by Jak1 and Tyk2 (39, 40). Phosphorylated STAT3 forms homodimers which translocate into the nucleus, bind to STAT-binding elements, and drive the expression of STAT-3-responsive genes. Among others, these include the suppressor of cytokine signaling 3 (SOCS-3) and the IL-1 receptor antagonist (IL-1RN) (**Figure 1**) (41): SOCS-3 subsequently inhibits mitogen-activated protein kinase activation, NFκB translocation into the nucleus, and the associated induction of pro-inflammatory gene expression (40). SOCS-3 also mediates Jak1-inhibition, resulting in feedback inhibition of the JAK1/Tyk2/STAT3 pathway (42).

In addition, production of IL-1RN, a decoy protein binding to the IL-1 receptor, blocks pro-inflammatory signaling normally initiated by binding of IL-1β to this receptor (**Figure 1**) (43).

Interleukin-10 also directly inhibits Th1 cell differentiation (by reducing IL-2, IL-12, and INF-γ production), limits effector T-cell function (by suppressing TNF-α, IL-1β, and IL-6 production), and promotes the development, expansion, and function of regulatory T cells (23, 44). For example, IL-10 is known to inhibit the expression of IL-4 and IL-13-responsive genes in monocytes and DCs by suppressing the activation of STAT6 (**Figure 1**) (45).

Interleukin-10/IL-10R signaling may also result in STAT1 and STAT5 phosphorylation in monocytes and Treg cells, but the interactions of STAT1 and STAT5 with other intracellular signaling events triggered by IL-10 are still unclear (42).

Because of its broadly anti-inflammatory effects, IL-10 is a highly interesting molecule for the treatment of allergic diseases, where affected patients mount exaggerated, immunoglobulin E (IgE)- and Th2-mediated immune responses against otherwise harmless environmental antigens.

In line with this, DC-derived autocrine IL-10 secretion was shown to suppress high-affinity IgE receptor Fc epsilon receptor I-dependent pro-inflammatory responses (46), suggesting that increased IL-10 production by DCs during allergy immunotherapy may reduce inflammatory responses to the allergen (47).

Up to now, numerous studies support the importance of IL-10 produced by either Treg or Tr1 cells (48, 49), IL-10-producing regulatory B cells (50), and lung DCs (4, 32, 51) in the modulation of allergic diseases. Among other findings, IL-10 production by murine lung DCs suppressed inflammation and promoted the establishment of allergen-specific tolerance (52). In line with its function in the suppression of lung inflammation, IL-10 expression has been reported in DCs located in both lung tissue and the intestine, suggesting IL-10 to fulfill an important role in maintaining local T-cell tolerance to common environmental antigens (32).

The importance of IL-10 in controlling allergic inflammation is further highlighted by its ability to decrease eosinophil survival

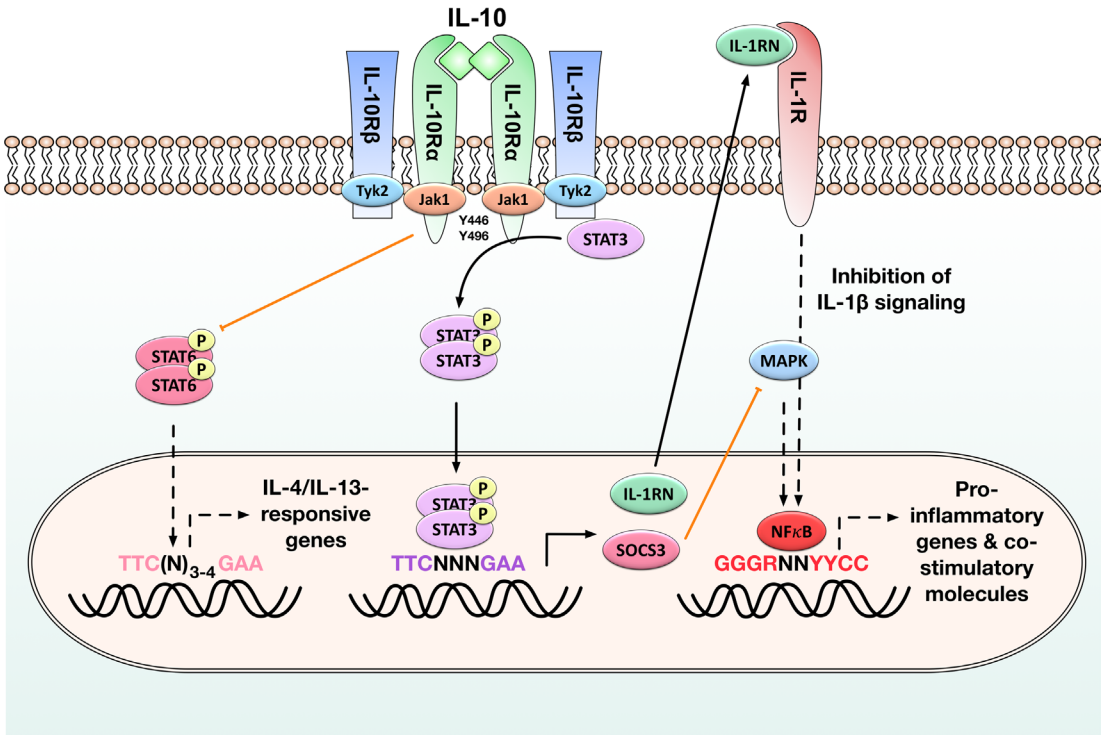


FIGURE 1 | Immune modulatory signaling in antigen-presenting cells (APCs) induced by binding of interleukin-10 (IL-10) to the IL-10 receptor. Activation of the IL-10/Janus kinase 1 (JAK1)/tyrosine kinase 2 (Tyk2)/signal transducer and activator of transcription 3 (STAT3) pathway in APCs results in the phosphorylation of STAT3 by the interleukin 10 receptor (IL-10R) complex and the subsequent translocation of STAT3 homodimers into the nucleus. There STAT3 homodimers bind to STAT-binding elements and drive the expression of STAT-3-responsive genes such as suppressor of cytokine signaling 3 (SOCS-3) and IL-1 receptor antagonist (IL-1RN): SOCS-3 efficiently inhibits mitogen-activated protein kinase (MAP kinase) activation, NFκB translocation into the nucleus, and the subsequent induction of pro-inflammatory gene expression, while the decoy protein IL-1RN suppresses pro-inflammatory signaling normally initiated by binding of IL-1β to its receptor. STAT3 activation also inhibits STAT6 activation and therefore the expression of IL-4/IL-13-responsive genes. By these events, IL-10 reduces the production of pro-inflammatory cytokines (IL-1β, IL-6, tumor necrosis factor alpha) and diminished expression of both major histocompatibility complex II and co-stimulatory molecules (CD80, CD83, CD86) on APCs. Black arrows: activation of the indicated signaling pathways, orange arrows: inhibition of the indicated signaling pathways, black dashed arrows: pathways normally induced by the indicated molecules that are inhibited in the presence of STAT3 activation. For more detailed information, see Section "IL-10 Is an Important Cytokine Limiting Excessive Immune Responses."

and IgE synthesis (53, 54). Indeed, IL-10 is often regarded as a key cytokine mediating tolerance in patients undergoing immunotherapy (55, 56).

Allergic Patients Show a Tendency to Produce Reduced IL-10 Levels upon Allergen Contact

In line with the importance of IL-10 in suppressing allergic responses, Akbari and colleagues reported that DCs from mice exposed to harmless inhaled antigens transiently produce IL-10 stimulating the development of IL-10-secreting, antigen-specific $CD4^+CD25^+Foxp3^+$ Tregs (32). Moreover, upon stimulation with the probiotic bacterium *Escherichia coli* 083, a lower expression and secretion of IL-10 was detected from monocyte-derived DCs (moDCs) derived from newborns of allergic mothers compared with cells derived from children with non-allergic mothers (57).

These results suggest that a reduced capacity to produce DC-derived IL-10 upon antigen contact may facilitate the development of allergic diseases by skewing immune responses toward the differentiation of Th2 cells and the development of childhood atopy and/or asthma (2).

Early studies showed that DC-derived IL-10 production is profoundly diminished in allergic rhinitis (AR) children regardless of the presence or absence of asthma, while DC-derived IL-12 secretion as well as T cell cytokine secretion were unchanged (2). These results suggest that atopic individuals have an intrinsic inability to upregulate DC-derived IL-10 production (2). In line with this, several studies reported diminished antigen-induced, peripheral blood mononuclear cell (PBMC)-derived IL-10 production in children (58, 59) and adults (31, 60, 61) with atopic disorders (AR, asthma, or atopic dermatitis).

In addition, several studies have reported that allergic patients show a tendency to produce reduced levels of IL-10 upon allergen exposure. For example, defects in IL-10-producing T cells have been implicated in the immunopathogenesis of airway allergy, resulting in Th2-mediated production of allergen-specific IgE and tissue eosinophilia (55, 62).

Moreover, Wei et al. reported that IL-10 levels in the supernatants of DCs from AR patients were significantly lower than those observed in healthy controls (63). Accordingly, Pilette and coworkers described not only local nasal DCs but also systemically circulating blood myeloid DCs (mDCs) from AR patients to exhibit reduced IL-10 and IL-12 expression after allergen provocation, while activated plasmacytoid DCs from these patients produced diminished amounts of interferon alpha (IFN-α) and triggered reduced levels of IL-10 from allogeneic $CD4^+$ T cells (64). Due to these changes in cytokine production mDCs from AR patients preferentially supported Th2-cell polarization, linking systemic DC dysfunction to biased T-cell responses and the failure to regulate T-cell-mediated responses to allergens seen in atopic patients (64).

While most studies suggest allergic patients to produce reduced levels of IL-10 compared with healthy individuals, some groups report contrary results: Lied and coworkers reported LPS-stimulated moDCs from atopic patients to produce significantly more IL-10 compared to non-atopic patients (65) and Frischmeyer-Guerrerio and colleagues described that mDCs from food allergic children produced greater quantities of IL-10 (66).

Successful Allergen-Specific Immunotherapy May Restore Reduced IL-10 Secretion in Allergic Patients

Since several studies have reported that allergic patients show a tendency to produce reduced levels of IL-10 upon allergen exposure (see above paragraph), restoring allergen-induced IL-10 secretion from DCs is one of the aims in allergen-specific immunotherapy (AIT).

Indeed, IL-10 is often regarded as a key cytokine mediating tolerance in patients undergoing immunotherapy (55, 56). Many of the observed beneficial immune alterations during AIT have been attributed to IL-10 production (67). Several studies reported increased levels of IL-10 in blood and affected tissues of patients that underwent AIT (4, 68, 69). However, the cellular source of AIT-induced IL-10 production seems to depend on the exact treatment modalities: for example, exposure to high doses of allergen was repeatedly shown to result in the induction of different IL-10-producing $CD4^+CD25^+Foxp3^+$ Treg subsets (70–72), while for patients receiving pollen AIT increased IL-10 production was reported in mucosal macrophages (69). Interestingly, in contrast to this, increased numbers of IL-10-producing B cells and monocytes were described in the peripheral blood of patients receiving bee venom AIT (70, 73).

While many of the beneficial effects of AIT-induced IL-10 production are attributed to the induction of allergen-specific, IL-10-producing $CD4^+CD25^+Foxp3^+$ Tregs (69–72), other cellular sources of IL-10 should not be ignored. In line with this, Kunz and coworkers recently described that the IL-10-dependent induction of allergen-specific tolerance by subcutaneous allergen injection resulted in increased IL-10 signals in T and B cells of both skin draining and mediastinal lymph nodes (74). Interestingly, tolerance induction could still be achieved when mice were unable to produce either T cell-, B cell-, T and B cell-, or DC-derived IL-10 (74). In contrast to this, tolerance induction was not possible if all hematopoetic cells were unable to produce IL-10 (74). Taken together, these results suggest a high degree of functional cellular redundancy in IL-10-mediated tolerance induction (74).

STRATEGIES TO INDUCE DC-DERIVED IL-10 SECRETION

We have seen that immature DCs can be used to as tolerogenic DCs to suppress antigen-specific T cell responses. A regulatory, IL-10-producing DC phenotype is of special interest for the treatment of allergic diseases where the treatment aim is to downregulate allergen-specific Th2 responses and the associated clinical symptoms (allergen-specific Th2-responses, IgE-mediated mast cell and basophil activation, allergic inflammation). The remainder of this review will summarize the strategies used to generate such IL-10-producing DCs with a focus on IL-10 treatment, IL-10 transduction, and the usage of both whole bacteria and bacteria-derived components (see also **Figures 2** and **3** and **Table 1**). Besides these strategies, several other factors have been described to promote the differentiation of IL-10-producing DC subsets in the context of allergies. Among others, these are endothelial splenic stroma (11), aryl hydrocarbon receptors (63), the phosphodiesterase-4 inhibitor roflumilast (75), and dexamethasone (16). Also induction of cyclic adenosine monophosphate was shown to result in upregulated expression of DC-derived IL-10, attenuated secretion of the pro-inflammatory cytokines TNF-α and IL-12, and impaired T cell stimulation by these DCs (76).

IL-10 Treatment

The simplest strategy to induce tolerogenic IL-10 DCs is to differentiate naive DCs in the presence of IL-10. Indeed, several studies reported IL-10-treated human or mouse DCs to induce antigen-specific anergy (99–101).

Immunologically, T cell tolerization (meaning the induction of antigen-specific $CD4^+CD25^+$ Treg cells) by IL-10-treated DCs requires a partially activated DC status commonly referred to as semimaturation (102, 103). In contrast to this, complete DC activation likely is more immunogenic than tolerogenic, resulting in the activation of effector T cell subsets (102, 103). This semimature status is characterized by high-expression levels of MHC, intermediate to low levels of co-stimulatory molecules, and a strongly reduced production of pro-inflammatory cytokines such as IL-12 (**Figure 2A**) (102). Moreover, the development of antigen-specific $CD4^+CD25^+Foxp3^+$ Treg cells, inhibiting allergic responses, was shown to be dependent on the presence of both IL-10 and an inducible co-stimulator (ICOS)–inducible co-stimulator ligand (ICOS-L) interaction provided by DCs (**Figure 2B**) (32).

Here, the suppression of Th2-responses by the induced regulatory T cells was repeatedly described to occur *via* a cell contact-dependent and antigen non-specific manner (48, 104, 105). For example, DCs treated with IL-10 were shown to induce $CD4^+$ T cells expressing the cytotoxic T lymphocyte-associated antigen 4 (CD152, CTLA4), capable of mediating tolerance in a

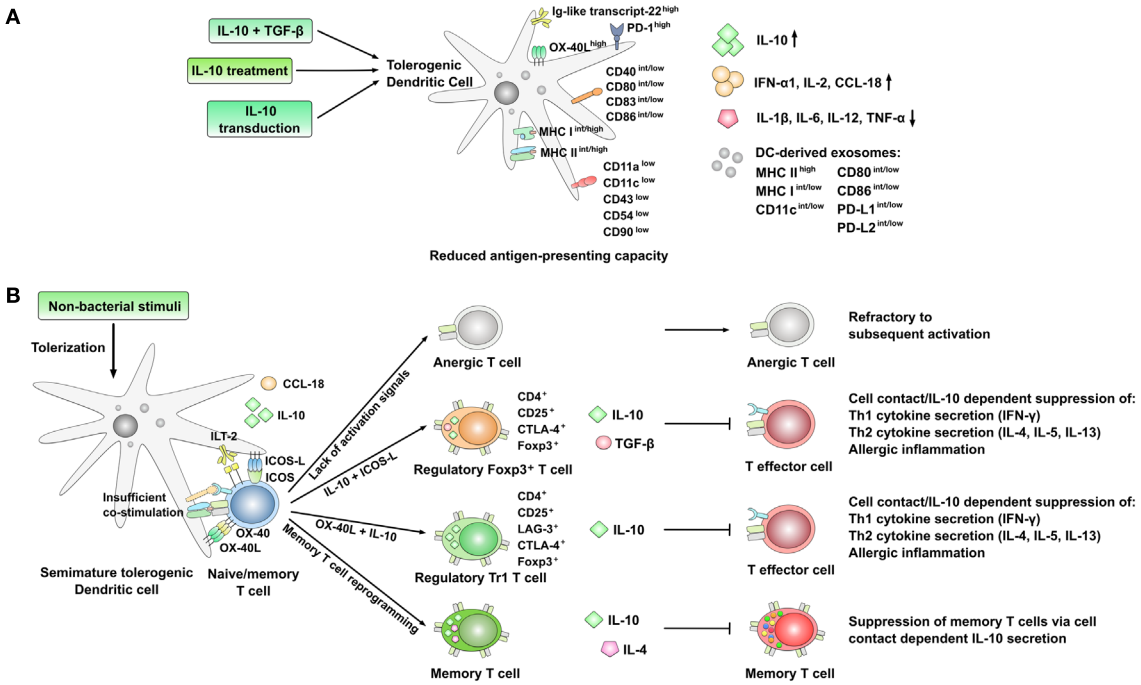


FIGURE 2 | Phenotype and immune modulatory effects of interleukin-10 (IL-10)-producing, semi-mature tolerogenic DCs. **(A)** Strategies resulting in the generation of IL-10-producing, semi-mature tolerogenic dendritic cells (DCs) with reduced expression of co-stimulatory molecules, cell adhesion molecules, and lower secretion of pro-inflammatory cytokines. Expression levels are indicated as follows: ^{low}: low expression, ^{int}: intermediate expression, ^{high}: high expression, $^{+}$: positive for the indicated molecule; arrow up: increased production, arrow down: decreased production. **(B)** Immune modulatory effects of tolerogenic DCs displaying a semi-mature DC phenotype. The lack of co-stimulation and antigen presentation results in the preferred induction of either anergic or regulatory T cell subsets which themselves are able to suppress both Th1- and Th2-responses. Also, the reprogramming of $CD4^+$ memory T cells into IL-10 and IL-4 co-producing Th0-like cells has been described.

cell contact-dependent manner (**Figure 2B**) (99, 106). In addition, some studies reported an increased expression of inhibitory molecules such as the Ig-like transcript-22/CD85 (77) on the surface of IL-10-treated DCs (**Figure 2A**).

The exact tolerogenic potential of IL-10-treated DCs seems to dependent on the experimental model used: while there are some reports suggesting that DCs treated with IL-10 may increase the secretion of Th2 cytokines (while suppressing Th1-responses) (107, 108), the majority of studies have described an efficient suppression of both mouse and human Th1- and Th2-responses by DCs treated with IL-10 (101).

The potency of IL-10-treated DCs to prevent the development of lung allergic responses in mice was demonstrated by Koya and colleagues (1). Koya et al. reported IL-10-treated DCs to suppress production of the Th2 cytokines IL-4, IL-5, and IL-13 *in vitro* and decrease both AHR and airway inflammation *in vivo* (1). Here, transfer of ovalbumin (OVA)-pulsed, IL-10-treated DCs into naive mice prevented the development of AHR, airway eosinophilia, reduced Th2 cytokine levels in bronchoalveolar lavage (BAL) fluid, and goblet cell metaplasia when challenged with the allergen (1).

In their hands, the IL-10-treated DCs displayed a tolerogenic phenotype, expressing lower levels of CD11c, CD80, and CD86, while producing lower amounts of IL-12 but significantly more IL-10 (**Figure 2A**) (1). Mechanistically, this endogenous IL-10 production of exogenously IL-10-treated DCs was shown to be required for their regulatory function since DCs from IL-10-deficient mice did not display regulatory function even when differentiated in the presence of IL-10 (1).

In accordance with these results, Li et al. did report IL-10-treated human DCs to induce tolerance in autologous T cells of patients with asthma (77). Phenotypically, the IL-10-treated DCs expressed reduced levels of the co-stimulatory and maturation markers CD86, human leukocyte antigen DR, and CD54, only modest reductions in CD40 and CD80, and reduced levels of the pro-inflammatory cytokines IL-6 and IL-12 (**Figure 2A**) (77). In contrast to this, expression levels of Ig-like transcript-22/CD85j, IFN-α1, IL-2, and IL-10 were strongly increased (**Figure 2A**) (77). In this context, the inhibitory receptor Ig-like transcript-22/CD85j was shown to have an important role both in the regulation of natural killer cells and T cells (109, 110) and the function of tolerogenic DCs (110).

In co-culture with autologous $CD4^+$ T cells IL-10-treated DCs inhibited Th2 cell differentiation and production of Th2-related cytokines (IL-4, IL-5, and IL-13) otherwise driven by immunostimulatory DCs differentiated in the presence of TNF-α (77). Moreover, treatment of DCs with IL-10 led to a significant outgrowth and activation of $CD4^+CD25^+LAG\text{-}3^+CTLA\text{-}4^+Foxp3^+$ IL-10-secreting Tr1-type Tregs, and resulted in allergen-specific induction of tolerance in a contact-dependent manner

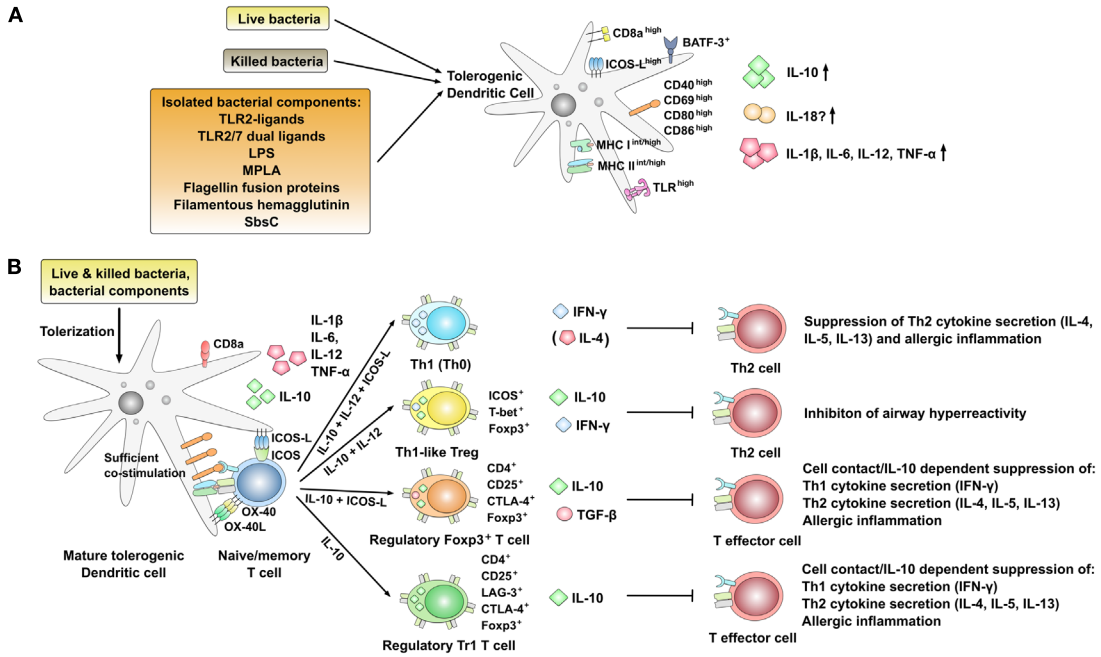


FIGURE 3 | Phenotype and immune modulatory effects of interleukin-10 (IL-10) producing, mature tolerogenic DCs co-producing pro-inflammatory cytokines. **(A)** Strategies using live or killed bacteria and bacterial components resulting in pro-tolerogenic dendritic cell (DC) phenotypes characterized by the expression of high levels of co-stimulatory molecules as well as the co-production of IL-10 and pro-inflammatory cytokines [IL-1β, IL-6, IL-12, tumor necrosis factor alpha (TNF-α)]. Expression levels are indicated as follows: ^{low}: low expression, ^{int}: intermediate expression, ^{high}: high expression, $^{+}$: positive for the indicated molecule; arrow up: increased production, arrow down: decreased production. **(B)** Regulation of T cell responses by DCs stimulated with bacteria or bacterial components inducing an IL-10-positive DC phenotype that is characterized by the co-production of IL-12 and high expression levels of co-stimulatory molecules. In this context, both the induction of different regulatory T cell subsets and Th1-biased effector cells have been described. $^{+}$: positive for the indicated molecule.

which was critically dependent on expression of IL-10 by DC (**Figure 2B**) (77).

Although the tolerogenic capacity of IL-10-treated DCs is well described, Bellinghausen et al. reported treatment of DCs with IL-10 alone (in contrast to the efficient suppression of Th1 responses by IL-10-treated DCs) to be insufficient for the suppression of Th2-responses (78). In their hands, the induction of regulatory T cells with the ability to suppress Th2 cytokine production required at least two signals: IL-10 plus TGF-β (78). In their experimental system, the suppressive capacity of the IL-10 plus TGF-β-induced regulatory T cells was shown to be antigen-unspecific and strongly dependent on both cell–cell contact and the surface molecule programmed death-1 (PD-1) (78). Interestingly, neutralization of either IL-10, CTLA-4, or TGF-β had only marginal effects on the suppressive capacity of the induced $CD4^+CD25^+$ Tregs (78). Here, incubation of T cells with IL-10 alone instead of IL-10-treated DC did not lead to the generation of inducible Tregs (iTregs), suggesting that additional signals provided by the tolerizing DC are necessary for the generation of iTregs (78).

One such factor might be DC-derived CC-chemokine ligand 18 (CCL18). When performing a genome-wide analysis of gene expression in co-cultures of $CD4^+$ T cells from patients with grass pollen allergy and either tolerogenic, IL-10-treated DCs or regular, mature allergen-pulsed DCs, Bellinghausen and coworkers could show that in DCs differentiated in the presence of IL-10 the only gene being upregulated was CCL18 (while many genes were downregulated) (79). These IL-10-treated, CCL18-producing DCs efficiently suppressed IL-13, IL-5, and TNF receptor superfamily member 4 (OX40) gene expression in CD4 T cell:DC co-cultures (79). Of note, exogenous addition of CCL18 to these co-cultures was sufficient to induce a similar inhibition of Th2 cytokine production compared to allergen-pulsed, IL-10-treated DCs (without affecting IFN-γ or IL-10 production) (79). In these co-cultures, neutralizing IL-10 did not reduce CCL18 production suggesting that factors other than IL-10 are involved in maintaining the enhanced CCL18 expression in IL-10-treated DCs (79). In a humanized mouse model of airway allergy, application of CCL18 inhibited airway reactivity and lung inflammation, preferentially attracting regulatory T cells over Th2 cells (79). Therefore, CCL18 was shown to be an important effector molecule of tolerogenic IL-10-treated DCs.

IL-10 Transduction

Besides differentiating DCs in the presence of IL-10, several studies have described transduction of DCs with the IL-10 gene to result in DCs with tolerogenic properties. Here, IL-10-transduced DCs were shown to induce long-lasting, antigen-specific tolerance by induction of regulatory T cells (**Figures 2A,B**) (4).

Henry and coworkers reported a single intratracheal injection of OVA-pulsed, IL-10-transduced DCs to prevent eosinophilic

TABLE 1 | Summary of the strategies reported in the literature to induce dendritic cell (DC)-derived interleukin-10 (IL-10) secretion.

Strategy		Reference	DC phenotype	DCs IL-10 positive?	Immunological effects of tolerogenic DCs	Suppression shown to be IL-10 dependent?
DC differentiation in the presence of IL-10		Koya et al. (1)	$CD11c^{low}$ $CD80^{low}$ $CD86^{low}$ Reduced IL-12 production	Yes	Suppression of Th2 cytokines IL-4, IL-5, and IL-13 *in vitro* Decrease of airway hyperreactivity (AHR) and airway inflammation *in vivo*	Yes
		Li et al. (77)	$CD86^{low}$ $HLA\text{-}DR^{low}$ $CD54^{low}$ $CD40^{int}$ $CD80^{int}$ Ig-like transcript-22/$CD85j^{high}$ reduced levels of IL-6 and IL-12	Yes	Suppression of Th2-differentiation and Th2-cytokine secretion Activation of $CD4^+CD25^+LAG\text{-}3^+$ $CTLA\text{-}4^+Foxp3^+$ IL-10-secreting Tregs Induction of allergen-specific tolerance	Yes and Treg cell contact dependent
		Bellinghausen et al. (78)	$IL\text{-}10^+$ $CTLA\text{-}4^+$ $TGF\text{-}\beta^+$	Yes	IL-10- and TGF-β-dependent induction of regulatory T cells suppressing Th2 cytokine production	No, but programmed death-1 dependent
		Bellinghausen et al. (79)	$CCL\text{-}18^+$	Yes	Suppression of IL-13, IL-5, and TNF receptor superfamily member 4 gene expression in $CD4^+$ T cell:DC co-cultures	No, but CCL18 dependent
DC transduction with IL-10	Lentiviral transduction with CMV-promoter	Henry et al. (4)	$CD40^{int}$ $MHC\ II^{int}$ $CD80^{int}$ $CD86^{int}$ $IL\text{-}12^{low}$	Yes	Prevention of eosinophilic airway inflammation, AHR, production of mucus, antigen-specific IgE and IgG1 antibody, and IL-4 production in a mouse model of experimental asthma	Yes and Treg dependent
	Plasmid vector	Nakagome et al. (80)	$CD11c^+$ $MHC\ II^{low}$	Yes	No induction of tolerogenic DCs or Treg, but overall suppression of function of CD11c antigen-presenting cells in the lung Prevention of eosinophilic airway inflammation *in vivo*	Not investigated
	Lentiviral transduction with DC-specific fascin promotor	Besche et al. (3)	$MHC\ II^{int}$ $CD86^{int}$ Unaltered IL-6 mRNA Lower IL-12p40 mRNA levels	Yes	Inhibition of ear swelling in mouse model of hapten-induced contact hypersensitivity	Not investigated
DC-derived exosomes		Kim et al. (81)	$MHC\ II^{high}$ $MHC\ I^{int}$ $CD11c^{int}$ $CD80^{int}$ $CD86^{int}$	No	Suppression of delayed-type hypersensitivity responses and murine collagen-induced arthritis	No, but via MHC II-dependent pathway
		Ruffner et al. (82)	IA/IE^{high} $H\text{-}2k^{b\ int}$ $CD80^{low}$ $CD86^{int}$ $PD\text{-}L1^{int}$ $PD\text{-}L2^{low}$ $IL\text{-}12p70^-$ $IL\text{-}23^-$ $IL\text{-}6^+$	Not determined	Suppression of delayed-type hypersensitivity responses	CD80 and CD86 dependent

(*Continued*)

TABLE 1 | Continued

Strategy		Reference	DC phenotype	DCs IL-10 positive?	Immunological effects of tolerogenic DCs	Suppression shown to be IL-10 dependent?
Bacteria	*Helicobacter pylori (live/extract)*	Engler et al. (83)	$BATF3^{+}$ $CD103^{+}$ $CD11b^{+}$	Yes	Suppression of airway inflammation in a mouse model of allergic asthma	Yes, and IL18 basic leucine zipper ATF-like 3 (BATF3) dependent
	Escherichia coli 083	Súkeníková et al. (57)	$CD83^{high}$ IDO^{high} TNF-α^{+} $IL\text{-}6^{+}$	Yes	Increased expression of IL-10 and IL-17A in CD4 T cells	Not investigated
	Bacillus Calmette–Guérin	Bilenki et al. (84)	$CD8a^{high}$ $CD80^{high}$ $CD86^{high}$ $CD40^{high}$ $IL\text{-}12^{+}$ $TLR2^{high}$, $TLR4^{high}$, $TLR9^{high}$	Yes	Suppression of allergic airway eosinophilia, mucus overproduction, IgE production, and Th2 cytokine production *in vivo*	Yes, also IL-12 dependent
	Listeria monocytogenes	Stock et al. (85)	$CD8a^{+}$ $IL\text{-}12^{+}$	Yes	Induction of Th1-like $ICOS^{+}$ $Foxp3^{+}$T-bet^{+} Tregs co-producing both IL-10 and interferon gamma (IFN-γ)	Yes
	Clamydia	Han et al. (27)	$CD8^{high}$ ICOS-L^{high} $IL\text{-}10^{high}$ $IL\text{-}12^{high}$	Yes	Inhibition of allergen-specific Th2 cell differentiation *in vitro* Inhibition of systemic and cutaneous eosinophilia *in vivo*	Yes, also IL-12 and ICOS-L dependent
Bacterial extracts	Heat killed *E. coli*	Pochard et al. (86)	$IA^{b\ high}$ $CD40^{int}$ $CD80^{high}$ $CD86^{high}$ $IL\text{-}12^{+}$	Yes	Suppression of peanut-induced Th2 cytokine production and proliferation and induction of IFN-γ from mouse T cells	No, but myeloid differentiation primary response 88 (MyD88)/ TIR-domain-containing adapter-inducing interferon-β, IL-12/IL23 p40, and IFN-γ dependent
Isolated bacterial components (TLR ligands)	Pam_3CSK_4	Tsai et al. (87)	Not investigated	Not investigated	Induction of $CD8^{+}CD25^{+}$ $Foxp3^{+}$ Tregs that inhibited *Dermatophagoides pteronyssinus* 2-induced IL-4 production *in vitro*	Not investigated
	Dual TLR2/7-ligands	Laiño et al. (88)	$IL\text{-}1\beta^{low}$ $IL\text{-}6^{+}$	Yes	Suppression of Th2 cytokine secretion and DNP-induced, IgE- and Ag-specific mast cell degranulation *in vitro* Suppression of allergen-specific IgE production *in vivo*	Not investigated
	LPS	Ahrens et al. (89)	$CD40^{high}$ $CD80^{high}$ $CD86^{high}$ $IL\text{-}1\beta^{+}$ $IL\text{-}12^{+}$ TNF-α^{+}	Yes	Suppression of Th2 cytokine production and induction of Tr1-like cells *in vitro* No suppression of ovalbumin (OVA)-induced asthma *in vivo*	Yes
	LPS (plus IL-10 treatment)	Wakkach et al. (90)	$CD11c^{low}$ $B220^{-}$ $CD45RB^{+}$	Yes	Increased eosinophilic airway inflammation and AHR, IL-5, and IL-13 secretion in bronchoalveolar lavage fluid in a mouse model of OVA-induced asthma	Not investigated
	Monophosphoryl lipid A	Schülke et al. (91)	$CD40^{+}$ $IL\text{-}1\beta^{+}$ $IL\text{-}6^{+}$ TNF-α^{+}	Yes	Boosting of OVA-specific IL-4 and IL-5 secretion, suppression of IFN-γ secretion in bone marrow-derived DC: DO11.10 $CD4^{+}$ T cell co-cultures	Not investigated

(*Continued*)

TABLE 1 | Continued

Strategy		Reference	DC phenotype	DCs IL-10 positive?	Immunological effects of tolerogenic DCs	Suppression shown to be IL-10 dependent?
	Flagellin fusion proteins	Schülke et al. (92–96)	$CD40^{+}$ $CD69^{+}$ $CD80/86^{+}$ $B7\text{-}H1^{+}$ $B7\text{-}H4^{+}$ $IL\text{-}6^{high}$	Yes	Suppression of Th1 and Th2 responses *in vitro* Suppression of sensitization and OVA-induced intestinal allergy *in vivo*	Yes
Isolated bacterial component (non TLR-ligands)	Flamentous hemagglutinin	McGuirk et al. (97)	$MHC\ II^{int}$ $CD40^{int}$ $CD80^{int}$ $CD86^{int}$ $CCR5^{int}$ $IL\text{-}12^{low}$ $CCL3^{low}$	Yes	Suppression of Th1 cell proliferation and cytokine secretion, but not Th2-responses *via* differentiation of Tr1 Tregs	Yes
	SbsC:Bet v 1 fusion proten	Gerstmayr et al. (98)	$CD40^{int}$ $CD80^{int}$ $CD86^{int}$ $IL\text{-}12^{+}$	Yes	Induction of IL-10-producing $CD25^{+}Foxp3^{+}CTLA\text{-}4^{+}$ Th0/regulatory T cells co-producing IFN-γ and IL-4	Not investigated, but IL-12 dependent

Expression levels are indicated as follows: ^{low}: low expression; ^{int}: intermediate expression; ^{high}: high expression; $^{+}$: positive for the investigated molecule but no expression level specified.

airway inflammation, AHR, production of mucus, antigen-specific IgE and immunoglobulin G1 (IgG1) antibodies, and IL-4 as well as IFN-γ production in a mouse model of experimental asthma (**Figure 2B**) (4). These effects were shown to also depend on non-DC-derived IL-10 since IL-10-deficient mice treated with IL-10-transduced wild-type DCs were less well protected (4). Phenotypically, IL-10-transduced DCs displayed intermediate levels of cell surface maturation markers MHC II, CD40, CD80, and CD86 and secreted high amounts of IL-10, but no IL-12 (**Figure 2A**) (4). In contact with allergen-specific T cells, these semi-mature DCs induced both differentiation and proliferation of antigen-specific $CD4^{+}CD25^{+}Foxp3^{+}$ IL-10-producing regulatory T cells in the mediastinal lymph nodes of animals treated with IL-10-transduced DCs (4). These effects were shown to be antigen-specific, since IL-10-transduced DCs, primed with the major house dust mite allergen *Dermatophagoides pteronyssinus* peptidase 1 (Der p 1), did not protect against OVA-induced airway allergy (4).

In line with these results, Nakagome et al. reported IL-10 gene delivery by plasmid transfer to suppress OVA-induced eosinophilic airway inflammation and AHR, suppressing the overall function of $CD11c^{+}$ lung APCs in terms of antigen-presenting capacity, cytokine production, and transport of antigen to lymph nodes resulting in reduced Th2 responses (80).

In an attempt to optimize DC-derived IL-10 transduction, Besche et al. showed that the usage of the DC-specific fascin promoter for IL-10 overexpression in bone marrow-derived DCs (BM-DCs) to result in the generation of $IL\text{-}10^{+}IL\text{-}6^{+}$ DCs with reduced IL-12p40 mRNA expression (3). *In vivo* application of these IL-10-transduced BM-DCs efficiently inhibited ear swelling responses in a mouse model of hapten-induced contact hypersensitivity (3).

Exosomes from IL-10-Treated DCs

Besides secreting immune modulatory cytokines, IL-10-treated DCs were also shown to secret exosomes with immune modulating capacity involved in the suppression of inflammatory and autoimmune responses (**Figure 2A**) (81).

Kim et al. reported exosomes isolated from either BM-DCs transduced *ex vivo* with an adenovirus expressing the IL-10 gene or BM-DCs treated with recombinant murine IL-10 protein to express high levels of MHC II, moderate levels of MHC I, CD11c, CD80, and CD86 on their surface (**Figure 2A**) (81). Upon periarticular administration, these exosomes were shown to suppress delayed-type hypersensitivity responses within both injected and untreated contralateral joints, while systemic injection suppressed the onset of murine collagen-induced arthritis and reduced the severity of established arthritis in a mouse model (**Figure 2B**) (81). Here, administration of isolated exosomes had comparable effects to the application of IL-10 transduced DC (81). Mechanistically, the suppressive capacity of the exosomes was shown to depend on surface expression of MHC II (81). The authors speculated that these exosomes may be able to bind and possibly fuse with endogenous cells (macrophages, or APCs) to subsequently modulate their activity (81).

These results were confirmed by Ruffner and colleagues which demonstrated that IL-10 treatment generates both DCs with a

pro-tolerogenic phenotype and a population of immunosuppressive exosomes (82). Treatment of DC with IL-10 significantly downregulated surface expression of MHC I, MHC II, CD80, CD86, and programmed death ligand 2 (PD-L2) (**Figure 2A**) (82). In addition to the modified co-stimulatory profile of IL-10-treated DCs, exosomes derived from these DCs were shown to also contain reduced surface levels of CD80, PD-L1, and PD-L2 (**Figure 2A**) (82). Here, the suppressive capacity of both IL-10-treated DCs and exosomes derived from these cells in a mouse model of delayed-type hypersensitivity was shown to depend on CD80 and CD86, but not PD-L1 and PD-L2 expression (82).

Live Bacteria

Several studies have described the potential of different bacteria to induce DC-derived IL-10 production (27, 84). Among others, *E. coli* 083 (57), *Helicobacter pylori* (83), *Clamydia* (27), *Listeria monocytogenes* (85), *Mycobacterium vaccae* (111), and *Bacillus* Calmette–Guérin (BCG) (84) were reported to induce DC-derived IL-10 secretion with immune modulatory potential for the treatment of allergic diseases. Of note, in contrast to IL-10-treated DCs, many of the available studies suggest that stimulation with either bacteria (live or killed) or isolated bacterial components induces both anti-inflammatory IL-10- and pro-inflammatory, Th1-promoting IL-12 secretion often alongside an enhanced expression of co-stimulatory molecules on the stimulated DCs (**Figure 3A**) (27, 84, 86, 98, 112).

Here, IL-10 secretion induced by bacteria can either prevent excessive inflammatory responses or suppress immune responses otherwise directed against the bacterium (27). In theory, this IL-10 induction by bacteria may be used to modulate immune responses in the host to unrelated antigens such as allergens (27).

In line with this, epidemiological and experimental studies revealed a strong inverse relationship between chronic *H. pylori* infection (which induces IL-10 secretion from DCs) and the risk of developing allergic asthma, hay fever, or eczema (113–115). Here, Engler and colleagues further investigated the mechanism underlying the protective effects of *H. pylori* (83). They reported extracts of *H. pylori* to prevent allergen-induced airway hyperresponsiveness, bronchoalveolar eosinophilia, pulmonary inflammation, and Th2 cytokine production in a mouse asthma model (**Figure 3B**) (83). Mechanistically, this suppression of Th2-responses was shown to require a heat-sensitive *H. pylori* component (possibly the *H. pylori* persistence determinants γ-glutamyl-transpeptidase GGT and the vacuolating cytotoxin VacA) and the production of IL-10 by basic leucine zipper ATF-like 3 (BATF3)-dependent CD103 and CD11b positive DCs infiltrating the lungs of protected animals (**Figure 3A**) (83). Moreover, both IL-18 and BATF3 were critically required for *H. pylori*-mediated protection against allergic responses (83). In contrast to this, suppression of Th2-responses was independent of regulatory T cells since antibody-mediated depletion of $CD25^+$ Tregs had no effect on the suppression of Th2-responses (83). Interestingly, *in vitro* BM-DC-derived IL-10 secretion induced by *H. pylori* extracts was shown to depend on myeloid differentiation primary response 88 (MyD88) and toll-like receptor (TLR)2- but not TLR4-signaling (83).

Súkeníková et al. reported *E. coli* 083 to also induce increased gene expression and secretion of IL-10 in DC of newborns of healthy mothers in comparison to DCs derived from newbornes from allergic mothers (57). This higher IL-10 production was associated with lower levels of IL-4, IL-13, IFN-γ, IL-17A, and IL-22 in DC:$CD4^+$ T cell co-cultures (**Figure 3B**) (57).

In line with these results, infection of mouse DCs with BCG resulted in a significant enhancement of both IL-10 and IL-12 production (84). Interestingly, BCG-stimulated DCs were characterized by a higher surface expression of CD8a, co-stimulatory molecules CD80, CD86, and CD40, and TLRs (**Figure 3A**) (84). Here, adoptive transfer of DCs from BCG-infected mice, but not DCs from naive mice, significantly inhibited established allergic airway eosinophilia, mucus overproduction, IgE production, and Th2 cytokine production (**Figure 3B**) (84). These protective effects of BCG-infected DCs were reversed by the application of either IL-10- or IL-12-neutralizing antibodies, showing both cytokines to be involved in the suppression of the allergic response (84).

Stock et al. described *L. monocytogenes* to induce $CD8a^+$ DCs co-producing both IL-10 and IL-12 (**Figure 3A**) (85). These DCs mediated the differentiation of $ICOS^+Foxp3^+$ T-box transcription factor TBX21 (T-bet)$^+$ Th1-like $CD4^+CD25^+$ Treg cells that themselves produced both IL-10 and the Th1 cytokine IFN-γ (**Figure 3B**) (85). Therefore, these cells combined features of both regulatory T cells and Th1 cells (85).

Han et al. reported the adoptive transfer of $CD8^+$ICOS ligand (ICOS-L)$^+$IL-10^+IL-12^+ DCs isolated from *Chlamydia*-infected mice (**Figure 3A**), but not those from naive mice, to inhibit OVA-induced systemic and cutaneous eosinophilia after intranasal challenge with OVA (27). *In vitro* DCs from *Chlamydia*-infected mice were shown to inhibit allergen-specific Th2 cell differentiation while promoting Th1 responses in an IL-10-, IL-12-, and ICOS-L-dependent way (**Figure 3B**) (27).

Taken together, these results show that in addition to inducing Th1-priming DCs, infection with different bacteria can result in the differentiation of tolerogenic, IL-10-producing DC subsets characterized by both high expression levels of co-stimulatory molecules and the co-production of pro-inflammatory cytokines (**Figure 3A**). Mechanistically, these more activated tolerogenic DCs were shown to suppress allergen-specific Th2-responses via the induction of either Th1, Th1-like Tregs, $CD4^+CD25^+Foxp3^+$ Treg, or Tr1 cells (**Figure 3B**).

Aside from results obtained with live bacteria, also bacterial extracts and heat killed bacteria were described to suppress allergen-specific Th2-responses. Here, both IL-10-dependent and -independent mechanisms of immune modulation were described.

For example, Pochard et al. reported the addition of heat killed *E. coli* to peanut-pulsed DCs to suppress both peanut-induced secretion of the Th2 cytokines IL-4, IL-5, IL-13, and T cell proliferation while increasing IFN-γ production in a MyD88/TIR-domain-containing adapter-inducing interferon-β-dependent manner (86). Although stimulation of the DCs with heat killed *E. coli* did trigger DC-derived, TLR4-dependent IL-10 secretion, these effects of were not influenced by neutralization of IL-10 but shown to be dependent of IL-12/IL23 p40 and IFN-γ secretion

(86). Therefore, the suppression of Th2-responses described by Pochard et al. is mediated by a TLR-mediated enhancement of Th1-responses, which in turn downregulate Th2-responses *via* IL-12 production (86).

Bacterial Components

Besides whole bacteria and bacterial extracts, which are complex mixtures of different, potentially immune modulating components, some single bacterial components have been investigated for their DC tolerizing potential in the context of allergies (**Figure 3A**). For differentiation purposes, these components will be divided into TLR-ligands and non-TLR-ligands.

TLR Ligands

Bacteria- and virus-derived pathogen-associated molecular patterns are sensed by pathogen-recognition receptors and induce innate and subsequent adaptive immune responses. Due to their intrinsic capacity to activate innate immune cells, TLR ligands are interesting immune modulating components for the treatment of allergic diseases. Here, TLR2-, TLR3-, TLR4-, and TLR5-ligands have been described to induce tolerogenic DC subsets.

Bacterial lipopeptides such as the TLR2/6-ligand Pam_2CysK_4 have repeatedly been shown to induce tolerogenic DC and regulatory T cell responses (**Figures 3A,B**) (87, 116, 117). In addition, TLR2-ligands have the potential to induce a Th1-promoting cytokine milieu, enhance Ag presentation of endogenous peptides by DCs (117), and suppress IL-5, IL-13, and IFN-γ responses from human house dust mite-allergic patients (**Figure 3B**) (118).

Here, chemical conjugation of the TLR2-ligand Pam_3CysK_4 to OVA-derived $CD8^+$ T cell peptide sequences resulted in a rapid and enhanced uptake in DCs (119). Moreover, dual TLR2/7-ligands combining the TLR2-ligand Pam_2CysK_4 and the synthetic TLR7-ligand CL264 into a single molecule were shown to induce strongly activated mDCs co-producing IL-10 and pro-inflammatory IL-6 (**Figure 3A**) (88). *In vitro*, these mDCs suppressed both DNP-induced, IgE- and Ag-dependent mast cell degranulation and IL-5 secretion from OVA-specific DO11.10 $CD4^+$ TC (**Figure 3B**) (88). *In vivo* application of one of these ligands, CL531, was found to suppress allergen-specific IgE production in a mouse model of OVA-induced intestinal allergy, suggesting that such TLR2/7-ligands have the potential to induce Th1-biased immune modulation *in vivo* (88).

Ahrens et al. described LPS stimulation to strongly enhance IL-10 production from mouse BM-DCs (**Figure 3A**). In co-culture with allergen-specific naive $CD4^+$ T cells, LPS-stimulated BM-DCs suppressed the secretion of Th1 and Th2 cytokines in an IL-10-dependent manner (89). Here, LPS priming of BM-DCs resulted in the differentiation of a Tr1-like T-cell subset upon co-culture of the primed DCs with naive T cells (89). Accordingly, Wakkach et al. reported LPS-, but not CpG-stimulation, to induce enhanced IL-10 secretion from IL-10-treated, $CD11c^{low}B220^-CD45RB^+$ DCs (**Figure 3A**) (90). However, the suppressive capacity of LPS-primed BM-DC *in vitro* did not translate into suppression of allergic airway disease *in vivo* since intranasal administration of these LPS + IL-10-primed BM-DCs into mice was unable to prevent allergic airway inflammation in a mouse model of OVA-induced asthma (89). Here, vaccination with theseBM-DCs led to an even stronger eosinophilic airway inflammation and AHR accompanied by significantly increased levels of IL-5 and IL-13 in BAL fluid (89).

While LPS cannot be applied in humans due to its high toxicity and pyrogenicity, monophosphoryl lipid A (MPLA), a detoxified TLR4-ligand derived from *Salmonella minnesota*, is already applied as adjuvant in several vaccine formulations. Here, vaccines containing MPLA have been licensed or are in phase III trials including Fendrix (hepatitis B), Cervarix (human papillomavirus-16 and human papillomavirus-18), and RTS,S (malaria) (120–122). For the treatment of allergies, MPLA mixed with grass pollen extract was shown to result in enhanced production of IFN-γ and reduce the production of IL-5 in PBMC from grass pollen-allergic patients (123). In line with these results, MPLA was shown to induce mouse BM-DC activation (CD40 upregulation) and secretion of both pro- (IL-1β, IL-6, TNF-α) and anti-inflammatory (IL-10) cytokines *in vitro* (**Figure 3A**) (91). Of note, when MPLA-stimulated mouse BM-DCs were co-cultured with DO11.10 $CD4^+$ T cells *in vitro* MPLA was shown to boost OVA-specific IL-4 and IL-5 secretion while dose-dependently suppressing IFN-γ secretion displaying a discrepancy between the results obtained *in vitro* and in clinical trials (91).

In addition, synthetic oligodeoxynucleotides containing CpG motifs (TLR9-ligands) either alone (124–126) or chemically linked to allergens (127–129) have been described to promote Th1 cytokine responses and decrease synthesis of IgE antibodies in allergic individuals. While these constructs were shown to induce the production of IL-12 and IL-18 from human moDCs (125) and induce IL-12, IFN-α, IFN-γ, IL-6, and IL-10 secretion from PBMCs (126), up to now no DC-derived IL-10 secretion has been reported upon application of these CpG-based vaccines.

In our own studies, we evaluated the induction of IL-10-producing mDCs using fusion proteins consisting of the recombinant TLR5-ligand flagellin A (rFlaA) from *L. monocytogenes* and either OVA from hen's egg as a model allergen (rFlaA:OVA) (92–94), the major mugwort allergen *Artemisia vulgaris* allergen 1 (Art v1) (rFlaA:Artv1) (95), or the major birch pollen allergen *Betula verrucosa* allergen 1 (Bet v 1) (rFlaA:Betv1) (96). Such fusion proteins efficiently target $TLR5^+$ immune cells, e.g., DCs, macrophages, and epithelial cells which take up, process, and present the fused antigen in the context of the flagellin-mediated cell activation.

Mouse bone marrow-derived mDCs stimulated with such fusion proteins were strongly activated [upregulation of CD40, CD69, CD80, CD86, programmed cell death 1 ligand 2 (B7-DC, CD273), PD-L1], displayed highly increased levels of the target receptor TLR5 on their cell surface, and secreted both pro- (IL-1β, IL-6) and anti-inflammatory (IL-10) cytokines (**Figure 3A**) (93, 95, 96). Interestingly, the non-fused mixture of both components (flagellin and allergen) did not have a comparable effect (93, 95, 96).

When co-cultured with allergen-specific $CD4^+$ T cells, these DCs efficiently suppressed both allergen-induced Th1 and Th2 cytokine secretion *in vitro* (**Figure 3B**) (93, 95, 96). In this experimental setting, the flagellin fusion protein-mediated suppression

of both Th1- and Th2-cytokine secretion was dependent on IL-10, since this effect was reversed when using either IL-10-neutralizing antibodies or IL-10-deficient mDC as APCs for the co-cultures (93).

In vivo vaccination with the rFlaA:OVA fusion protein efficiently protected mice from OVA-induced gastrointestinal allergy. Here, vaccination with rFlaA:OVA either intraperitoneal (93) or intranasal (92) was sufficient to prevent intestinal allergy induced by continuous challenge with OVA-containing food pellets. Interestingly, core body temperature, body weight, food up-take, and symptom scores were significantly improved in rFlaA:OVA-treated mice in comparison to the respective control groups (92, 93). This protective effect was associated with a reduction of Th2 cytokines in intestinal homogenates, suppression of systemic T cell immune responses, suppression of OVA-specific IgE-, and induction of OVA-specific IgG2a-responses (93). Vaccination with rFlaA and OVA alone or provided as a mixture did neither prevent allergic sensitization nor improve allergy symptom scores.

Mechanistically, stimulation of mDCs with flagellin fusion proteins was shown to result in a stronger uptake into mDCs (92, 96) accompanied by an increased resistance to microsomal digestion (92, 96). Interestingly, stimulation with rFlaA:Betv1 was shown to result in an increased metabolic activity of the stimulated mDCs characterized by a high rate of glycolysis followed by lactic acid fermentation, known as the Warburg effect (130). Further analysis suggested an activation of the mammalian target of rapamycin 1 complex in mDCs stimulated with the fusion protein (96). In this context, recent studies suggest that mTOR is not only a master regulator of cell metabolic function but also regulates innate immune responses (131).

Inhibition of the mTOR complex by pre-treatment of the cells with rapamycin dose-dependently suppressed the induction of anti-inflammatory IL-10 secretion by rFlaA:Betv1, but not pro-inflammatory cytokine secretion (IL-1β and IL-6). These findings show that interestingly, the immune-modulatory cytokine secretion, and therefore the DC tolerizing capacity, of this vaccine candidate was linked to the activation of mDC metabolism. Similar results were obtained for a fusion construct consisting of FlaA and the major mugwort allergen Art v 1 (95).

Non-TLR Ligands with DC-Tolerizing Potential

In the context of allergy treatment, some non-TLR ligands have been reported to induce DCs with tolerizing potential. Here, *Bordetella pertussis*-derived filamentous hemagglutinin was shown to induce the production of IL-10 by DCs promoting the differentiation of $CD4^+CD25^+CCR5^{high}CD28^{low}CTLA\text{-}4^{low}$ IL-10- and IL-5-co-producing Tr1 cells (97).

Moreover, Gerstmayr and colleagues generated a recombinant fusion protein of a bacterial surface (S-layer) protein of *Geobacillus stearothermophilus* (SbsC) and the major birch pollen allergen Bet v 1 as a vaccine candidate to improve the treatment of birch pollen allergy. The SbsC:Bet v 1 fusion protein displayed reduced mediator-releasing capacity, while both preserving Bet v 1-T cell epitopes, and the potency to induce IFN-γ and IL-10 production in Bet v 1-specific Th2-biased T cell clones (98). DCs stimulated with the fusion protein were shown to have a semi-mature phenotype characterized by enhanced expression of CD40, CD80, and CD86 which were still lower than the levels induced by stimulation with LPS (**Figure 3A**) (98). Moreover, the SbsC:Bet v 1 fusion protein strongly increased DC-derived IL-10 and IL-12 secretion (98). Functionally, DCs matured with SbsC:Bet v 1 induced the IL-12- and IFN-γ-dependent differentiation of naive T cells into IFN-γ-producing T cells co-producing IL-4, suggesting a Th0 phenotype (**Figure 3B**) (98). In addition, naive T cells also differentiated into IL-10-producing $CD4^+CD25^+Foxp3^+C$ LTA-4^+ regulatory T cells capable of active suppression, thus promoting the simultaneous differentiation of Th0/Th1 cells and regulatory T cells (**Figure 3B**) (98).

OTHER FACTORS CONTRIBUTING TO THE INHIBITORY CAPACITY OF DCs

Although many studies described the suppressive capacity of IL-10 secreting DCs, several other factors were reported to also mediate suppression of T cell responses without inducing DC-derived IL-10 production. Here, LPS- or polyriboinosinic-polyribocytidilic acid-induced production of IL-1β, indoleamine 2,3-dioxygenase (132), transforming growth factor-beta 1, vitamin D3 (133), corticosteroids, cyclosporine (134), as well as neuropeptides have been used to generate tolerogenic DCs (135, 136). Therefore, although the induction of IL-10 reproducibly leads to a tolerogenic phenotype of the induced DCs, other agents may also be used to generate DCs able to suppress T cells responses.

SUMMARY

Because of their potent T cell stimulatory as well as regulatory properties DCs have become a highly attractive tool in vaccine development to modulate antigen-specific immune responses.

While for cancer treatment and vaccination against infectious diseases the efficient induction of adaptive immune responses against the target antigens is the desired outcome when applying DC-based vaccination approaches, the therapy of autoimmune diseases, transplantat rejection, allergic reactions, or the control of chronic inflammation aims to induce DCs with tolerogenic properties.

Comparing IL-10-producing DCs induced either by IL-10-treatment or IL-10-transduction to IL-10-secreting DCs generated by stimulation with bacteria and bacterial components has revealed striking differences in the phenotype of the induced DCs and therefore the mechanism of tolerization: tolerogenic DCs generated by non-bacterial stimuli are arrested in an immature or semi-mature state, characterized by the production of reduced amounts of cytokines that promote T cell activation such as IL-12 and IL-6, a reduced capacity to present exogenous antigens, and the expression of lower amounts of co-stimulatory molecules.

In contrast to this, treatment of DCs with live or killed bacteria as well as isolated bacterial components results in the induction of both anti-inflammatory IL-10- as well as pro-inflammatory,

Th1-promoting, IL-12 secretion often paralleled by an enhanced expression of co-stimulatory molecules on the stimulated DCs. This induction of Th1-priming, tolerogenic DCs generated by strongly activating stimuli was shown to suppress allergen-specific Th2-responses *via* the induction of either Th1-like Tregs, $CD4^+CD25^+Foxp3^+$ Tregs, or Tr1 cells.

Therefore, while displaying the ability to directly suppress both Th1- and Th2-responses by different mechanisms, IL-10-producing DCs can efficiently modulate antigen-specific-specific immune responses *via* the induction of T cell subsets with regulatory functions. This makes IL-10-producing DCs promising therapeutics to improve the treatment of allergic diseases.

Over the past few years, we have started to understand the complex molecular mechanisms underlying the immune modulatory capacity of IL-10-producing DCs, identifying novel DC-derived factors that drive T cell tolerization such as DC-derived exosomes, CCL18, and inhibitory molecules like CTLA-4, OX40, Ig-like transcript-22/CD85, or PD-1.

Future studies will undoubtedly further increase our knowledge about the underlying immunological mechanisms allowing us to both refine and improve the application of DC-based vaccination approaches.

AUTHOR CONTRIBUTIONS

SS performed all research, prepared all the figures and tables, and wrote the manuscript.

FUNDING

This work has been funded by the budget of the Paul-Ehrlich-Institut, Langen, Germany.

REFERENCES

1. Koya T, Matsuda H, Takeda K, Matsubara S, Miyahara N, Balhorn A, et al. IL-10-treated dendritic cells decrease airway hyperresponsiveness and airway inflammation in mice. *J Allergy ClinImmunol* (2007) 119:1241–50. doi:10.1016/j.jaci.2007.01.039
2. Gentile DA, Schreiber R, Howe-Adams J, Trecki J, Patel A, Angelini B, et al. Diminished dendritic cell interleukin 10 production in atopic children. *Ann Allergy Asthma Immunol* (2004) 92:538–44. doi:10.1016/S1081-1206(10)61761-9
3. Besche V, Wiechmann N, Castor T, Trojandt S, Höhn Y, Kunkel H, et al. Dendritic cells lentivirally engineered to overexpress interleukin-10 inhibit contact hypersensitivity responses, despite their partial activation induced by transduction-associated physical stress. *J Gene Med* (2010) 12(3):231–43. doi:10.1002/jgm.1436
4. Henry E, Desmet CJ, Garzé V, Fiévez L, Bedoret D, Heirman C, et al. Dendritic cells genetically engineered to express IL-10 induce long-lasting antigen-specific tolerance in experimental asthma. *J Immunol* (2008) 181:7230–42. doi:10.4049/jimmunol.181.10.7230
5. Steinman RM. The dendritic cell system and its role in immunogenicity. *Annu Rev Immunol* (1991) 9:271–96. doi:10.1146/annurev.iy.09.040191.001415
6. Schuurhuis DH, Fu N, Ossendorp F, Melief CJM. Ins and outs of dendritic cells. *Int Arch Allergy Immunol* (2006) 140:53–72. doi:10.1159/000092002
7. Lanzavecchia A. Mechanisms of antigen uptake for presentation. *Curr Opin Immunol* (1996) 8:348–54. doi:10.1016/S0952-7915(96)80124-5
8. Banchereau J, Briere F, Caux C, Davoust J, Lebecque S, Liu YJ, et al. Immunobiology of dendritic cells. *Annu Rev Immunol* (2000) 18:767–811. doi:10.1146/annurev.immunol.18.1.767
9. Chan VW, Kothakota S, Rohan MC, Panganiban-Lustan L, Gardner JP, Wachowicz MS, et al. Secondary lymphoid-tissue chemokine (SLC) is chemotactic for mature dendritic cells. *Blood* (1999) 93:3610–6.
10. Sallusto F, Schaerli P, Loetscher P, Schaniel C, Lenig D, Mackay CR, et al. Rapid and coordinated switch in chemokine receptor expression during dendritic cell maturation. *Eur J Immunol* (1998) 28:2760–9. doi:10.1002/(SICI)1521-4141(199809)28:09<2760::AID-IMMU2760>3.0.CO;2-N
11. Xu X, Guo Z, Jiang X, Yao Y, Gao Q, Ding Y, et al. Regulatory dendritic cells program generation of interleukin-4-producing alternative memory CD4 T cells with suppressive activity. *Blood* (2011) 117:1218–27. doi:10.1182/blood-2010-05-285494
12. Guida G, Boita M, Scirelli T, Bommarito L, Heffler E, Badiu I, et al. Innate and lymphocytic response of birch-allergic patients before and after sublingual immunotherapy. *Allergy Asthma Proc* (2012) 33:411–5. doi:10.2500/aap.2012.33.3588
13. Steinman RM, Banchereau J. Taking dendritic cells into medicine. *Nature* (2007) 449:419–26. doi:10.1038/nature06175
14. Steinman RM, Hawiger D, Nussenzweig MC. Tolerogenic dendritic cells. *Annu Rev Immunol* (2003) 21:685–711. doi:10.1146/annurev.immunol.21.120601.141040
15. Li MO, Flavell RA. Contextual regulation of inflammation: a duet by transforming growth factor-beta and interleukin-10. *Immunity* (2008) 28:468–76. doi:10.1016/j.immuni.2008.03.003
16. Escobar A, Aguirre A, Guzmán MA, González R, Catalán D, Acuña-Castillo C, et al. Tolerogenic dendritic cells derived from donors with natural rubber latex allergy modulate allergen-specific T-cell responses and IgE production. *PLoS One* (2014) 9:e85930. doi:10.1371/journal.pone.0085930
17. Francis JN, Till SJ, Durham SR. Induction of IL-10+CD4+CD25+ T cells by grass pollen immunotherapy. *J Allergy Clin Immunol* (2003) 111:1255–61. doi:10.1067/mai.2003.1570
18. Mahnke K, Ring S, Bedke T, Karakhanova S, Enk AH. Interaction of regulatory T cells with antigen-presenting cells in health and disease. *Chem Immunol Allergy* (2008) 94:29–39. doi:10.1159/000154854
19. Pulendran B, Ahmed R. Translating innate immunity into immunological memory: implications for vaccine development. *Cell* (2006) 124:849–63. doi:10.1016/j.cell.2006.02.019
20. Moser M, Murphy KM. Dendritic cell regulation of TH1-TH2 development. *Nat Immunol* (2000) 1:199–205. doi:10.1038/79734
21. Wu K, Bi Y, Sun K, Xia J, Wang Y, Wang C. Suppression of allergic inflammation by allergen-DNA-modified dendritic cells depends on the induction of Foxp3+ regulatory T cells. *Scand J Immunol* (2008) 67:140–51. doi:10.1111/j.1365-3083.2007.02050.x
22. Couper KN, Blount DG, Riley EM. IL-10: the master regulator of immunity to infection. *J Immunol* (2008) 180:5771–7. doi:10.4049/jimmunol.180.9.5771
23. Moore KW, de Waal Malefyt R, Coffman RL, O'Garra A. Interleukin-10 and the interleukin-10 receptor. *Annu Rev Immunol* (2001) 19:683–765. doi:10.1146/annurev.immunol.19.1.683
24. Saraiva M, O'Garra A. The regulation of IL-10 production by immune cells. *Nat Rev Immunol* (2010) 10:170–81. doi:10.1038/nri2711
25. Clausen BE, Girard-Madoux MJH. IL-10 control of dendritic cells in the skin. *Oncoimmunology* (2014) 2:e23186–23184. doi:10.4161/onci.23186
26. Vieira P, de Waal Malefyt R, Dang MN, Johnson KE, Kastelein R, Fiorentino DF, et al. Isolation and expression of human cytokine synthesis inhibitory factor cDNA clones: homology to Epstein-Barr virus open reading frame BCRFI. *Proc Natl Acad Sci U S A* (1991) 88:1172–6. doi:10.1073/pnas.88.4.1172
27. Han X, Wang S, Fan Y, Yang J, Jiao L, Qiu H, et al. Chlamydia infection induces ICOS ligand-expressing and IL-10-producing dendritic cells that can inhibit airway inflammation and mucus overproduction elicited by allergen challenge in BALB/c mice. *J Immunol* (2006) 176:5232–9. doi:10.4049/jimmunol.176.9.5232
28. Levings MK, Gregori S, Tresoldi E, Cazzaniga S, Bonini C, Roncarolo MG. Differentiation of Tr1 cells by immature dendritic cells requires IL-10

but not CD25+CD4+ Tr cells. *Blood* (2005) 105:1162–9. doi:10.1182/blood-2004-03-1211
29. Groux H, Bigler M, de Vries JE, Roncarolo MG. Interleukin-10 induces a long-term antigen-specific anergic state in human CD4+ T cells. *J Exp Med* (1996) 184:19–29. doi:10.1084/jem.184.1.19
30. Pacciani V, Gregori S, Chini L, Corrente S, Chianca M, Moschese V, et al. Induction of anergic allergen-specific suppressor T cells using tolerogenic dendritic cells derived from children with allergies to house dust mites. *J Allergy Clin Immunol* (2010) 125:727–36. doi:10.1016/j.jaci.2009.12.004
31. Oh J-W, Seroogy CM, Meyer EH, Akbari O, Berry G, Fathman CG, et al. CD4 T-helper cells engineered to produce IL-10 prevent allergen-induced airway hyperreactivity and inflammation. *J Allergy ClinImmunol* (2002) 110:460–8. doi:10.1067/mai.2002.127512
32. Akbari O, DeKruyff RH, Umetsu DT. Pulmonary dendritic cells producing IL-10 mediate tolerance induced by respiratory exposure to antigen. *Nat Immunol* (2001) 2:725–31. doi:10.1038/90667
33. Kearley J, Barker JE, Robinson DS, Lloyd CM. Resolution of airway inflammation and hyperreactivity after in vivo transfer of CD4+CD25+ regulatory T cells is interleukin 10 dependent. *J Exp Med* (2005) 202:1539–47. doi:10.1084/jem.20051166
34. Berg DJ, Leach MW, Kühn R, Rajewsky K, Müller W, Davidson NJ, et al. Interleukin 10 but not interleukin 4 is a natural suppressant of cutaneous inflammatory responses. *J Exp Med* (1995) 182:99–108. doi:10.1084/jem.182.1.99
35. Pestka S, Krause CD, Sarkar D, Walter MR, Shi Y, Fisher PB. Interleukin-10 and related cytokines and receptors. *Annu Rev Immunol* (2004) 22:929–79. doi:10.1146/annurev.immunol.22.012703.104622
36. de Waal Malefyt R, Haanen J, Spits H, Roncarolo MG, Velde te A, Figdor C, et al. Interleukin 10 (IL-10) and viral IL-10 strongly reduce antigen-specific human T cell proliferation by diminishing the antigen-presenting capacity of monocytes via downregulation of class II major histocompatibility complex expression. *J Exp Med* (1991) 174:915–24. doi:10.1084/jem.174.4.915
37. Kotenko SV, Krause CD, Izotova LS, Pollack BP, Wu W, Pestka S. Identification and functional characterization of a second chain of the interleukin-10 receptor complex. *EMBO J* (1997) 16:5894–903. doi:10.1093/emboj/16.19.5894
38. Finbloom DS, Winestock KD. IL-10 induces the tyrosine phosphorylation of tyk2 and Jak1 and the differential assembly of STAT1 alpha and STAT3 complexes in human T cells and monocytes. *J Immunol* (1995) 155:1079–90.
39. Weber-Nordt RM, Riley JK, Greenlund AC, Moore KW, Darnell JE, Schreiber RD. Stat3 recruitment by two distinct ligand-induced, tyrosine-phosphorylated docking sites in the interleukin-10 receptor intracellular domain. *J Biol Chem* (1996) 271:27954–61. doi:10.1074/jbc.271.44.27954
40. Berti FCB, Pereira APL, Cebinelli GCM, Trugilo KP, Brajão de Oliveira K. The role of interleukin 10 in human papilloma virus infection and progression to cervical carcinoma. *Cytokine Growth Factor Rev* (2017) 34:1–13. doi:10.1016/j.cytogfr.2017.03.002
41. Berlato C, Cassatella MA, Kinjyo I, Gatto L, Yoshimura A, Bazzoni F. Involvement of suppressor of cytokine signaling-3 as a mediator of the inhibitory effects of IL-10 on lipopolysaccharide-induced macrophage activation. *J Immunol* (2002) 168:6404–11. doi:10.4049/jimmunol.168.12.6404
42. Williams L, Bradley L, Smith A, Foxwell B. Signal transducer and activator of transcription 3 is the dominant mediator of the anti-inflammatory effects of IL-10 in human macrophages. *J Immunol* (2004) 172:567–76. doi:10.4049/jimmunol.172.1.567
43. Dinarello CA. The interleukin-1 family: 10 years of discovery. *FASEB J* (1994) 8(15):1314–25. doi:10.1096/fasebj.8.15.8001745
44. Biswas PS, Pedicord V, Ploss A, Menet E, Leiner I, Pamer EG. Pathogen-specific CD8 T cell responses are directly inhibited by IL-10. *J Immunol* (2007) 179:4520–8. doi:10.4049/jimmunol.179.7.4520
45. Dickensheets HL, Donnelly RP. IFN-gamma and IL-10 inhibit induction of IL-1 receptor type I and type II gene expression by IL-4 and IL-13 in human monocytes. *J Immunol* (1997) 159:6226–33.
46. Le T, Tversky J, Chichester KL, Bieneman AP, Huang S-K, Wood RA, et al. Interferons modulate Fc epsilon RI-dependent production of autoregulatory IL-10 by circulating human monocytoid dendritic cells. *J Allergy Clin Immunol* (2009) 123:217–23. doi:10.1016/j.jaci.2008.09.013
47. Frischmeyer-Guerrerio PA, Keet CA, Guerrerio AL, Chichester KL, Bieneman AP, Hamilton RG, et al. Modulation of dendritic cell innate and adaptive immune functions by oral and sublingual immunotherapy. *Clin Immunol* (2014) 155:47–59. doi:10.1016/j.clim.2014.08.006
48. Bellinghausen I, Knop J, Saloga J. The role of interleukin 10 in the regulation of allergic immune responses. *Int Arch Allergy Immunol* (2001) 126:97–101. doi:10.1159/000049499
49. Akbari O, Umetsu DT. Role of regulatory dendritic cells in allergy and asthma. *Curr Opin Allergy Clin Immunol* (2004) 4:533–8. doi:10.1097/00130832-200412000-00010
50. Braza F, Chesne J, Castagnet S, Magnan A, Brouard S. Regulatory functions of B cells in allergic diseases. *Allergy* (2014) 69:1454–63. doi:10.1111/all.12490
51. Faith A, Singh N, Chevretton E, Roberts D, Lee T, Corrigan C, et al. Counter regulation of the high affinity IgE receptor, FcepsilonRI, on human airway dendritic cells by IL-4 and IL-10. *Allergy* (2009) 64:1602–7. doi:10.1111/j.1398-9995.2009.02060.x
52. Akbari O, Freeman GJ, Meyer EH, Greenfield EA, Chang TT, Sharpe AH, et al. Antigen-specific regulatory T cells develop via the ICOS-ICOS-ligand pathway and inhibit allergen-induced airway hyperreactivity. *Nat Med* (2002) 8:1024–32. doi:10.1038/nm745
53. Enk AH, Angeloni VL, Udey MC, Katz SI. Inhibition of langerhans cell antigen-presenting function by IL-10. A role for IL-10 in induction of tolerance. *J Immunol* (1993) 151:2390–8.
54. Punnonen J, de Waal Malefyt R, van Vlasselaer P, Gauchat JF, de Vries JE. IL-10 and viral IL-10 prevent IL-4-induced IgE synthesis by inhibiting the accessory cell function of monocytes. *J Immunol* (1993) 151:1280–9.
55. Francis JN, James LK, Paraskevopoulos G, Wong C, Calderon MA, Durham SR, et al. Grass pollen immunotherapy: IL-10 induction and suppression of late responses precedes IgG4 inhibitory antibody activity. *J Allergy Clin Immunol* (2008) 121:1120–5.e2. doi:10.1016/j.jaci.2008.01.072
56. Bohle B, Kinaciyan T, Gerstmayr M, Radakovics A, Jahn-Schmid B, Ebner C. Sublingual immunotherapy induces IL-10-producing T regulatory cells, allergen-specific T-cell tolerance, and immune deviation. *J Allergy ClinImmunol* (2007) 120:707–13. doi:10.1016/j.jaci.2007.06.013
57. Súkeníková L, Černý V, Novotná O, Petrásková P, Boráková K, Kolářová L, et al. Different capacity of in vitro generated myeloid dendritic cells of newborns of healthy and allergic mothers to respond to probiotic strain *E. coli* O83:K24:H31. *Immunol Lett* (2017) 189:82–9. doi:10.1016/j.imlet.2017.05.013
58. Niwa Y. Elevated RANTES levels in plasma or skin and decreased plasma IL-10 levels in subsets of patients with severe atopic dermatitis. *Arch Dermatol* (2000) 136:125–6. doi:10.1001/archderm.136.1.125
59. Stelmach I, Jerzynska J, Kuna P. A randomized, double-blind trial of the effect of glucocorticoid, antileukotriene and beta-agonist treatment on IL-10 serum levels in children with asthma. *Clin Exp Allergy* (2002) 32:264–9. doi:10.1046/j.1365-2222.2002.01286.x
60. Gentile DA, Patel A, Ollila C, Fireman P, Zeevi A, Doyle WJ, et al. Diminished IL-10 production in subjects with allergy after infection with influenza A virus. *J Allergy ClinImmunol* (1999) 103:1045–8. doi:10.1016/S0091-6749(99)70177-6
61. Tournoy KG, Kips JC, Pauwels RA. Endogenous interleukin-10 suppresses allergen-induced airway inflammation and nonspecific airway responsiveness. *Clin Exp Allergy* (2000) 30:775–83. doi:10.1046/j.1365-2222.2000.00838.x
62. Anderson AE, Mackerness KJ, Aizen M, Carr VA, Nguyen D, Pre Du F, et al. Seasonal changes in suppressive capacity of CD4+ CD25+ T cells from patients with hayfever are allergen-specific and may result in part from expansion of effector T cells among the CD25+ population. *Clin Exp Allergy* (2009) 39:1693–9. doi:10.1111/j.1365-2222.2009.03320.x
63. Wei P, Hu G-H, Kang H-Y, Yao H-B, Kou W, Liu H, et al. An aryl hydrocarbon receptor ligand acts on dendritic cells and T cells to suppress the Th17 response in allergic rhinitis patients. *Lab Invest* (2014) 94:528–35. doi:10.1038/labinvest.2014.8
64. Pilette C, Jacobson MR, Ratajczak C, Detry B, Banfield G, VanSnick J, et al. Aberrant dendritic cell function conditions Th2-cell polarization in allergic rhinitis. *Allergy* (2013) 68:312–21. doi:10.1111/all.12090

65. Lied GA, Vogelsang P, Berstad A, Appel S. Dendritic cell populations in patients with self-reported food hypersensitivity. *Int J Gen Med* (2011) 4:389–96. doi:10.2147/IJGM.S17655
66. Frischmeyer-Guerrerio PA, Guerrerio AL, Chichester KL, Bieneman AP, Hamilton RA, Wood RA, et al. Dendritic cell and T cell responses in children with food allergy. *Clin Exp Allergy* (2010) 41:61–71. doi:10.1111/j.1365-2222.2010.03606.x
67. Akdis CA, Akdis M. Mechanisms of immune tolerance to allergens: role of IL-10 and Tregs. *J Clin Invest* (2014) 124:4678–80. doi:10.1172/JCI78891
68. Nasser SM, Ying S, Meng Q, Kay AB, Ewan PW. Interleukin-10 levels increase in cutaneous biopsies of patients undergoing wasp venom immunotherapy. *Eur J Immunol* (2001) 31:3704–13. doi:10.1002/1521-4141(200112)31:12<3704::AID-IMMU3704>3.0.CO;2-3
69. Nouri-Aria KT, Wachholz PA, Francis JN, Jacobson MR, Walker SM, Wilcock LK, et al. Grass pollen immunotherapy induces mucosal and peripheral IL-10 responses and blocking IgG activity. *J Immunol* (2004) 172:3252–9. doi:10.4049/jimmunol.172.5.3252
70. Akdis CA, Blesken T, Akdis M, Wüthrich B, Blaser K. Role of interleukin 10 in specific immunotherapy. *J Clin Invest* (1998) 102:98–106. doi:10.1172/JCI2250
71. Jutel M, Akdis M, Budak F, Aebischer-Casaulta C, Wrzyszcz M, Blaser K, et al. IL-10 and TGF-beta cooperate in the regulatory T cell response to mucosal allergens in normal immunity and specific immunotherapy. *Eur J Immunol* (2003) 33:1205–14. doi:10.1002/eji.200322919
72. Möbs C, Slotosch C, Löffler H, Jakob T, Hertl M, Pfützner W. Birch pollen immunotherapy leads to differential induction of regulatory T cells and delayed helper T cell immune deviation. *J Immunol* (2010) 184:2194–203. doi:10.4049/jimmunol.0901379
73. van de Veen W, Stanic B, Yaman G, Wawrzyniak M, Söllner S, Akdis DG, et al. IgG4 production is confined to human IL-10-producing regulatory B cells that suppress antigen-specific immune responses. *J Allergy Clin Immunol* (2013) 131:1204–12. doi:10.1016/j.jaci.2013.01.014
74. Kunz S, Dolch A, Surianarayanan S, Dorn B, Bewersdorff M, Alessandrini F, et al. T cell derived IL-10 is dispensable for tolerance induction in a murine model of allergic airway inflammation. *Eur J Immunol* (2016) 46:2018–27. doi:10.1002/eji.201646319
75. Bros M, Montermann E, Cholaszczyńska A, Reske-Kunz AB. The phosphodiesterase 4 inhibitor roflumilast augments the Th17-promoting capability of dendritic cells by enhancing IL-23 production, and impairs their T cell stimulatory activity due to elevated IL-10. *Int Immunopharmacol* (2016) 35:174–84. doi:10.1016/j.intimp.2016.03.025
76. Kambayashi T, Wallin RP, Ljunggren HG. cAMP-elevating agents suppress dendritic cell function. *J Leukoc Biol* (2001) 70:903–10. doi:10.1189/jlb.70.6.903
77. Li X, Yang A, Huang H, Zhang X, Town J, Davis B, et al. Induction of type 2 T helper cell allergen tolerance by IL-10-differentiated regulatory dendritic cells. *Am J Respir Cell Mol Biol* (2010) 42:190–9. doi:10.1165/rcmb.2009-0023OC
78. Bellinghausen I, König B, Böttcher I, Knop J, Saloga J. Inhibition of human allergic T-helper type 2 immune responses by induced regulatory T cells requires the combination of interleukin-10-treated dendritic cells and transforming growth factor-beta for their induction. *Clin Exp Allergy* (2006) 36:1546–55. doi:10.1111/j.1365-2222.2006.02601.x
79. Bellinghausen I, Reuter S, Martin H, Maxeiner J, Luxemburger U, Türeci Ö, et al. Enhanced production of CCL18 by tolerogenic dendritic cells is associated with inhibition of allergic airway reactivity. *J Allergy Clin Immunol* (2012) 130:1384–93. doi:10.1016/j.jaci.2012.08.039
80. Nakagome K, Dohi M, Okunishi K, Komagata Y, Nagatani K, Tanaka R, et al. In vivo IL-10 gene delivery suppresses airway eosinophilia and hyperreactivity by down-regulating APC functions and migration without impairing the antigen-specific systemic immune response in a mouse model of allergic airway inflammation. *J Immunol* (2005) 174:6955–66. doi:10.4049/jimmunol.174.11.6955
81. Kim SH, Lechman ER, Bianco N, Menon R, Keravala A, Nash J, et al. Exosomes derived from IL-10-treated dendritic cells can suppress inflammation and collagen-induced arthritis. *J Immunol* (2005) 174:6440–8. doi:10.4049/jimmunol.174.10.6440
82. Ruffner MA, Kim SH, Bianco NR, Francisco LM, Sharpe AH, Robbins PD. B7-1/2, but not PD-L1/2 molecules, are required on IL-10-treated tolerogenic DC and DC-derived exosomes for in vivo function. *Eur J Immunol* (2009) 39:3084–90. doi:10.1002/eji.200939407
83. Engler DB, Reuter S, van Wijck Y, Urban S, Kyburz A, Maxeiner J, et al. Effective treatment of allergic airway inflammation with *Helicobacter pylori* immunomodulators requires BATF3-dependent dendritic cells and IL-10. *Proc Natl Acad Sci U S A* (2014) 111:11810–5. doi:10.1073/pnas.1410579111
84. Bilenki L, Gao X, Wang S, Yang J, Fan Y, Han X, et al. Dendritic cells from mycobacteria-infected mice inhibits established allergic airway inflammatory responses to ragweed via IL-10- and IL-12-secreting mechanisms. *J Immunol* (2010) 184:7288–96. doi:10.4049/jimmunol.0902829
85. Stock P, Akbari O, Berry G, Freeman GJ, DeKruyff RH, Umetsu DT. Induction of T helper type 1-like regulatory cells that express Foxp3 and protect against airway hyper-reactivity. *Nat Immunol* (2004) 5:1149–56. doi:10.1038/ni1122
86. Pochard P, Vickery B, Berin MC, Grishin A, Sampson HA, Caplan M, et al. Targeting toll-like receptors on dendritic cells modifies the T(H)2 response to peanut allergens in vitro. *J Allergy Clin Immunol* (2010) 126:92–7.e5. doi:10.1016/j.jaci.2010.04.003
87. Tsai Y-G, Yang KD, Niu D-M, Chien J-W, Lin C-Y. TLR2 agonists enhance CD8+Foxp3+ regulatory T cells and suppress Th2 immune responses during allergen immunotherapy. *J Immunol* (2010) 184:7229–37. doi:10.4049/jimmunol.1000083
88. Laiño J, Wangorsch A, Blanco F, Wolfheimer S, Krause M, Flaczyk A, et al. Targeting of immune cells by Dual TLR2/7 ligands suppresses features of allergic Th2 immune responses in mice. *J Immunol Res* (2017) 2017:7983217. doi:10.1155/2017/7983217
89. Ahrens B, Freund T, Rha R-D, Dittrich A-M, Quarcoo D, Hutloff A, et al. Lipopolysaccharide stimulation of dendritic cells induces interleukin-10 producing allergen-specific T cells in vitro but fails to prevent allergic airway disease. *Exp Lung Res* (2009) 35:307–23. doi:10.1080/01902140802709460
90. Wakkach A, Fournier N, Brun V, Breittmayer J-P, Cottrez F, Groux H. Characterization of dendritic cells that induce tolerance and T regulatory 1 cell differentiation in vivo. *Immunity* (2003) 18:605–17. doi:10.1016/S1074-7613(03)00113-4
91. Schülke S, Flaczyk A, Vogel L, Gaudenzio N, Angers I, Löschner B, et al. MPLA shows attenuated pro-inflammatory properties and diminished capacity to activate mast cells in comparison with LPS. *Allergy* (2015) 70(10):1259–68. doi:10.1111/all.12675
92. Schülke S, Wolfheimer S, Gadermaier G, Wangorsch A, Siebeneicher S, Briza P, et al. Prevention of intestinal allergy in mice by rflaA:Ova is associated with enforced antigen processing and TLR5-dependent IL-10 secretion by mDC. *PLoS One* (2014) 9:e87822. doi:10.1371/journal.pone.0087822
93. Schülke S, Burggraf M, Waibler Z, Wangorsch A, Wolfheimer S, Kalinke U, et al. A fusion protein of flagellin and ovalbumin suppresses the TH2 response and prevents murine intestinal allergy. *J Allergy Clin Immunol* (2011) 128:1340–8.e12. doi:10.1016/j.jaci.2011.07.036
94. Schülke S, Waibler Z, Mende M-S, Zoccatelli G, Vieths S, Toda M, et al. Fusion protein of TLR5-ligand and allergen potentiates activation and IL-10 secretion in murine myeloid DC. *Mol Immunol* (2010) 48:341–50. doi:10.1016/j.molimm.2010.07.006
95. Schülke S, Kuttich K, Wolfheimer S, Duschek N, Wangorsch A, Reuter A, et al. Conjugation of wildtype and hypoallergenic mugwort allergen Art v 1 to flagellin induces IL-10-DC and suppresses allergen-specific TH2-responses in vivo. *Sci Rep* (2017) 7:11782. doi:10.1038/s41598-017-11972-w
96. Schülke S, Fiedler A-H, Ann-Christine J, Flaczyk A, Wolfheimer S, Anke H, et al. Critical role of mammalian target of rapamycin for IL-10 DC induction by a flagellin FlaA-conjugate preventing allergic sensitization. *J Allergy ClinImmunol* (2017). doi:10.1016/j.jaci.2017.07.002
97. McGuirk P, McCann C, Mills KHG. Pathogen-specific T regulatory 1 cells induced in the respiratory tract by a bacterial molecule that stimulates interleukin 10 production by dendritic cells: a novel strategy for evasion of protective T helper type 1 responses by *Bordetella pertussis*. *J Exp Med* (2002) 195:221–31. doi:10.1084/jem.20011288

98. Gerstmayr M, Ilk N, Schabussova I, Jahn-Schmid B, Egelseer EM, Sleytr UB, et al. A novel approach to specific allergy treatment: the recombinant allergen-S-layer fusion protein rSbsC-Bet v 1 matures dendritic cells that prime Th0/Th1 and IL-10-producing regulatory T cells. *J Immunol* (2007) 179:7270–5. doi:10.4049/jimmunol.179.11.7270
99. Steinbrink K, Jonuleit H, Müller G, Schuler G, Knop J, Enk AH. Interleukin-10-treated human dendritic cells induce a melanoma-antigen-specific anergy in CD8(+) T cells resulting in a failure to lyse tumor cells. *Blood* (1999) 93:1634–42.
100. Steinbrink K, Wölfl M, Jonuleit H, Knop J, Enk AH. Induction of tolerance by IL-10-treated dendritic cells. *J Immunol* (1997) 159:4772–80.
101. Bellinghausen I, Brand U, Steinbrink K, Enk AH, Knop J, Saloga J. Inhibition of human allergic T-cell responses by IL-10-treated dendritic cells: differences from hydrocortisone-treated dendritic cells. *J Allergy Clin Immunol* (2001) 108:242–9. doi:10.1067/mai.2001.117177
102. Lutz MB, Schuler G. Immature, semi-mature and fully mature dendritic cells: which signals induce tolerance or immunity? *Trends Immunol* (2002) 23:445–9. doi:10.1016/S1471-4906(02)02281-0
103. Reis e Sousa C. Dendritic cells in a mature age. *Nat Rev Immunol* (2006) 6:476–83. doi:10.1038/nri1845
104. Steinbrink K, Graulich E, Kubsch S, Knop J, Enk AH. CD4(+) and CD8(+) anergic T cells induced by interleukin-10-treated human dendritic cells display antigen-specific suppressor activity. *Blood* (2002) 99:2468–76. doi:10.1182/blood.V99.7.2468
105. Jonuleit H, Schmitt E, Schuler G, Knop J, Enk AH. Induction of interleukin 10-producing, nonproliferating CD4(+) T cells with regulatory properties by repetitive stimulation with allogeneic immature human dendritic cells. *J Exp Med* (2000) 192:1213–22. doi:10.1084/jem.192.9.1213
106. Sato K, Yamashita N, Baba M, Matsuyama T. Modified myeloid dendritic cells act as regulatory dendritic cells to induce anergic and regulatory T cells. *Blood* (2003) 101:3581–9. doi:10.1182/blood-2002-09-2712
107. De Smedt T, Van Mechelen M, De Becker G, Urbain J, Leo O, Moser M. Effect of interleukin-10 on dendritic cell maturation and function. *Eur J Immunol* (1997) 27:1229–35. doi:10.1002/eji.1830270526
108. Liu L, Rich BE, Inobe J, Chen W, Weiner HL. Induction of Th2 cell differentiation in the primary immune response: dendritic cells isolated from adherent cell culture treated with IL-10 prime naive CD4+ T cells to secrete IL-4. *Int Immunol* (1998) 10:1017–26. doi:10.1093/intimm/10.8.1017
109. Morel E, Bellón T. HLA class I molecules regulate IFN-gamma production induced in NK cells by target cells, viral products, or immature dendritic cells through the inhibitory receptor ILT2/CD85j. *J Immunol* (2008) 181:2368–81. doi:10.4049/jimmunol.181.4.2368
110. Young NT, Waller ECP, Patel R, Roghanian A, Austyn JM, Trowsdale J. The inhibitory receptor LILRB1 modulates the differentiation and regulatory potential of human dendritic cells. *Blood* (2008) 111:3090–6. doi:10.1182/blood-2007-05-089771
111. Adams VC, Hunt JRF, Martinelli R, Palmer R, Rook GAW, Brunet LR. *Mycobacterium vaccae* induces a population of pulmonary CD11c+ cells with regulatory potential in allergic mice. *Eur J Immunol* (2004) 34:631–8. doi:10.1002/eji.200324659
112. Schülke S, Vogel L, Junker A-C, Hanschmann K-M, Flaczyk A, Vieths S, et al. A fusion protein consisting of the vaccine adjuvant monophosphoryl lipid A and the allergen ovalbumin boosts allergen-specific Th1, Th2, and Th17 responses in vitro. *J Immunol Res* (2016) 2016:4156456–8. doi:10.1155/2016/4156456
113. Amberbir A, Medhin G, Erku W, Alem A, Simms R, Robinson K, et al. Effects of *Helicobacter pylori*, geohelminth infection and selected commensal bacteria on the risk of allergic disease and sensitization in 3-year-old Ethiopian children. *Clin Exp Allergy* (2011) 41:1422–30. doi:10.1111/j.1365-2222.2011.03831.x
114. Chen Y, Blaser MJ. *Helicobacter pylori* colonization is inversely associated with childhood asthma. *J Infect Dis* (2008) 198:553–60. doi:10.1086/590158
115. Arnold IC, Dehzad N, Reuter S, Martin H, Becher B, Taube C, et al. *Helicobacter pylori* infection prevents allergic asthma in mouse models through the induction of regulatory T cells. *J Clin Invest* (2011) 121:3088–93. doi:10.1172/JCI45041
116. Chen Q, Davidson TS, Huter EN, Shevach EM. Engagement of TLR2 does not reverse the suppressor function of mouse regulatory T cells, but promotes their survival. *J Immunol* (2009) 183:4458–66. doi:10.4049/jimmunol.0901465
117. DePaolo RW, Kamdar K, Khakpour S, Sugiura Y, Wang W, Jabri B. A specific role for TLR1 in protective T(H)17 immunity during mucosal infection. *J Exp Med* (2012) 209:1437–44. doi:10.1084/jem.20112339
118. Taylor RC, Richmond P, Upham JW. Toll-like receptor 2 ligands inhibit TH2 responses to mite allergen. *J Allergy Clin Immunol* (2006) 117:1148–54. doi:10.1016/j.jaci.2006.02.014
119. Khan S, Bijker MS, Weterings JJ, Tanke HJ, Adema GJ, van Hall T, et al. Distinct uptake mechanisms but similar intracellular processing of two different toll-like receptor ligand-peptide conjugates in dendritic cells. *J Biol Chem* (2007) 282:21145–59. doi:10.1074/jbc.M701705200
120. Kundi M. New hepatitis B vaccine formulated with an improved adjuvant system. *Expert Rev Vaccines* (2007) 6:133–40. doi:10.1586/14760584.6.2.133
121. Agnandji ST, Lell B, Soulanoudjingar SS, Fernandes JF, Abossolo BP, Conzelmann C, et al. First results of phase 3 trial of RTS,S/AS01 malaria vaccine in African children. *N Engl J Med* (2011) 365:1863–75. doi:10.1056/NEJMoa1102287
122. GlaxoSmithKline Vaccine HPV-007 Study Group, Romanowski B, de Borba PC, Naud PS, Roteli-Martins CM, De Carvalho NS, et al. Sustained efficacy and immunogenicity of the human papillomavirus (HPV)-16/18 AS04-adjuvanted vaccine: analysis of a randomised placebo-controlled trial up to 6.4 years. *Lancet* (2009) 374:1975–85. doi:10.1016/S0140-6736(09)61567-1
123. Puggioni F, Durham SR, Francis JN. Monophosphoryl lipid A (MPLR)* promotes allergen-induced immune deviation in favour of Th1 responses. *Allergy* (2005) 60:678–84. doi:10.1111/j.1398-9995.2005.00762.x
124. Parronchi P, Brugnolo F, Annunziato F, Manuelli C, Sampognaro S, Mavilia C, et al. Phosphorothioate oligodeoxynucleotides promote the in vitro development of human allergen-specific CD4+ T cells into Th1 effectors. *J Immunol* (1999) 163:5946–53.
125. Bohle B, Jahn-Schmid B, Maurer D, Kraft D, Ebner C. Oligodeoxynucleotides containing CpG motifs induce IL-12, IL-18 and IFN-gamma production in cells from allergic individuals and inhibit IgE synthesis in vitro. *Eur J Immunol* (1999) 29:2344–53. doi:10.1002/(SICI)1521-4141(199907)29:07<2344::AID-IMMU2344>3.0.CO;2-R
126. Horner AA, Widhopf GF, Burger JA, Takabayashi K, Cinman N, Ronaghy A, et al. Immunostimulatory DNA inhibits IL-4-dependent IgE synthesis by human B cells. *J Allergy ClinImmunol* (2001) 108:417–23. doi:10.1067/mai.2001.117795
127. Marshall JD, Abtahi S, Eiden JJ, Tuck S, Milley R, Haycock F, et al. Immunostimulatory sequence DNA linked to the Amb a 1 allergen promotes T(H)1 cytokine expression while downregulating T(H)2 cytokine expression in PBMCs from human patients with ragweed allergy. *J Allergy ClinImmunol* (2001) 108:191–7. doi:10.1067/mai.2001.116984
128. Tighe H, Takabayashi K, Schwartz D, Van Nest G, Tuck S, Eiden JJ, et al. Conjugation of immunostimulatory DNA to the short ragweed allergen Amb a 1 enhances its immunogenicity and reduces its allergenicity. *J Allergy ClinImmunol* (2000) 106:124–34. doi:10.1067/mai.2000.107927
129. Horner AA, Takabayashi K, Beck L, Sharma B, Zubeldia J, Baird S, et al. Optimized conjugation ratios lead to allergen immunostimulatory oligodeoxynucleotide conjugates with retained immunogenicity and minimal anaphylactogenicity. *J Allergy ClinImmunol* (2002) 110:413–20. doi:10.1067/mai.2002.126660
130. Powell JD, Pollizzi KN, Heikamp EB, Horton MR. Regulation of immune responses by mTOR. *Annu Rev Immunol* (2012) 30:39–68. doi:10.1146/annurev-immunol-020711-075024
131. Säemann MD, Haidinger M, Hecking M, Hörl WH, Weichhart T. The multifunctional role of mTOR in innate immunity: implications for transplant immunity. *Am J Transplant* (2009) 9:2655–61. doi:10.1111/j.1600-6143.2009.02832.x
132. Bubnoff Von D, Scheler M, Wilms H, Fimmers R, Bieber T. Identification of IDO-positive and IDO-negative human dendritic cells after activation by various proinflammatory stimuli. *J Immunol* (2011) 186:6701–9. doi:10.4049/jimmunol.1003151

133. Lyakh LA, Sanford M, Chekol S, Young HA, Roberts AB. TGF-beta and vitamin D3 utilize distinct pathways to suppress IL-12 production and modulate rapid differentiation of human monocytes into CD83+ dendritic cells. *J Immunol* (2005) 174:2061–70. doi:10.4049/jimmunol.174.4.2061
134. Ciesek S, Ringe BP, Strassburg CP, Klempnauer J, Manns MP, Wedemeyer H, et al. Effects of cyclosporine on human dendritic cell subsets. *Transplant Proc* (2005) 37:20–4. doi:10.1016/j.transproceed.2004.11.055
135. Delgado M, Chorny A, Ganea D, Gonzalez-Rey E. Vasoactive intestinal polypeptide induces regulatory dendritic cells that prevent acute graft versus host disease and leukemia relapse after bone marrow transplantation. *Ann N Y Acad Sci* (2006) 1070:226–32. doi:10.1196/annals.1317.019
136. de Jong EC, Vieira PL, Kalinski P, Kapsenberg ML. Corticosteroids inhibit the production of inflammatory mediators in immature monocyte-derived DC and induce the development of tolerogenic DC3. *J Leukoc Biol* (1999) 66:201–4. doi:10.1002/jlb.66.2.201

Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

MINI REVIEW
published: 20 June 2018
doi: 10.3389/fimmu.2018.01389

Interaction of Non-Specific Lipid-Transfer Proteins With Plant-Derived Lipids and Its Impact on Allergic Sensitization

Stephan Scheurer and Stefan Schülke*

Molecular Allergology, Paul-Ehrlich-Institut, Langen, Germany

Non-specific lipid-transfer proteins (nsLTPs) represent a family of ubiquitous plant proteins belonging to the prolamin superfamily. nsLTPs are characterized by a globular α-helical structure stabilized by four disulfide bonds and a hydrophobic cavity which acts as ligand-binding site for a broad spectrum of lipids and hydrophobic molecules. nsLTPs are involved in membrane biogenesis and in the adaption of plants to abiotic and biotic stress. They display antimicrobial activity by the ability to permeabilize the cell membrane of phytopathogens. Moreover, in the presence of lipids, nsLTPs are suggested to activate the plant immune system by a receptor-dependent mechanism. Additionally, nsLTPs from pollen and plant-derived food, in particular type 1 nsLTPs (9 kDa), are described as potent allergens. Within the nsLTP family Pru p 3 from peach is the clinically most relevant allergen which can cause genuine food allergy and frequently elicits severe clinical reactions. So far, the allergenic properties of nsLTPs are attributed to both their low molecular mass and their high thermal and proteolytic stability which allow them to reach the immune system in a biological intact form. Recently, the interaction of nsLTPs with lipids has been suggested to increase their allergenic properties and to promote the allergic sensitization to these proteins. This review will summarize the current knowledge on diversity of lipid ligands of plant LTPs, and illustrate recent studies performed with allergenic nsLTPs to investigate the effect of lipid binding on the structural modification and IgE-binding properties of proteins, and finally the potential effect on the innate immune responses.

Keywords: non-specific lipid-transfer protein, allergen, lipid, fatty acid, (glycero)phospholipid, CD1d, invariant natural killer T cell

OPEN ACCESS

Edited by:
Nestor González Roldán, Allergobiochemie, Forschungszentrum Borstel, Germany

Reviewed by:
Liam OMahony, Swiss Institute of Allergy and Asthma Research (SIAF), Switzerland
Domingo Barber, Universidad CEU San Pablo, Spain
Oscar Palomares, Complutense University of Madrid, Spain

****Correspondence:***
Stephan Scheurer
stephan.scheurer@pei.de

Specialty section:
This article was submitted to Molecular Innate Immunity, a section of the journal Frontiers in Immunology

Received: *26 April 2018*
Accepted: *05 June 2018*
Published: *20 June 2018*

Citation:
Scheurer S and Schülke S (2018) Interaction of Non-Specific Lipid-Transfer Proteins With Plant-Derived Lipids and Its Impact on Allergic Sensitization. Front. Immunol. 9:1389. doi: 10.3389/fimmu.2018.01389

INTRODUCTION

The incidence of allergies is increasing worldwide (1, 2). However, the reason why only few protein families cause aero- and food allergy is still unsolved. There is increasing evidence that the allergenicity of proteins cannot be solely attributed to structural and physicochemical properties of the molecules itself (3). Factors which determine the allergenicity of food proteins are highly diverse, comprising also matrix effects, and the interaction of allergens with natural ligands affecting their antigenicity and immunogenicity (3, 4). Upon ingestion of food, allergens and lipids, including fatty acids (FAs), glycerolipids [triglycerides and phospholipids, e.g., phosphatidylcholine (PC)], or sphingolipids, are delivered to the immune system, either admixed in an unbound form or as a complex when lipophilic

molecules act as natural ligands of allergens. In both cases, the presence of lipids was suggested to modify the allergenicity and the immune-modulating properties of the antigens (5).

IMPACT OF LIPIDS ON THE SENSITIZATION CAPACITY OF FOOD ALLERGENS

Several studies indicated lipid mediators derived from bacterial and pollen contaminants and dietary lipids to modulate the allergenic properties of proteins (6). Phospholipid binding to Ber e 1 (major Brazil nut allergen, 2S albumin) *via* the engagement of IL-4-producing invariant (i)NKT cells (7, 8), binding of dietary medium-chain triglycerides to peanut proteins, PC binding to the Bet v 1-homologous apple allergen Mal d 1 (9), phosphatidylglycerol (PG) binding to Ara h 1 (7S globulin from peanut) and Sin a 2 (11S globulin from mustard) (10), and preincubation of peanut extract and allergen Ana o 2 from cashew with oleic acid (11) were reported to change the immunogenicity of the respective allergens by either increasing allergen stability and IgE-reactivity or stimulating allergen absorption and subsequent T_H2 responses. Here, lipid extracts from nuts favor allergen-induced inflammatory responses by increasing IL-4/IL-10 ratios and IL-1β secretion from human monocyte-derived DCs (moDCs) (11). Moreover, purified peanut-derived lipids applied together with either Arh 1 or Ara h 2 were reported to trigger pro-inflammatory responses (IL-8, IL-6, and TNF-α secretion) while inhibiting anti-inflammatory IL-10 release from human keratinocytes (12).

In summary, lipophilic components, mainly the oil or lipid fraction from nuts, have immune-modulating capacity and can affect the allergic response. However, in contrast to the mode of the molecular interaction, (1) the impact of lipids on the allergic sensitization and (2) the effect of lipid binding to food allergens on their immunogenicity and allergenicity are less investigated. Lipid-binding properties have been suggested for numerous food allergens, e.g., the Bet v 1-family (Ara h 8, peanut, Mal d 1, apple), lipocalins (Bos d 5, bovine milk), 2S albumins (Ber a 1, brazil nut, Sin a 1, mustard), 7S and 11S globulins (Ara h 1 and Sin a 2), alpha-lactalbumin Bos d 4, vicilin Gly m 5 (soybean), legumin-like protein Ana o 2 (cashew), as well as plant non-specific lipid-transfer proteins (nsLTPs) (5, 6). From an allergological viewpoint, the interaction of lipids with nsLTPs is of special interest because these proteins frequently induce severe clinical symptoms. Therefore, this review will focus on the interaction of different lipids with nsLTPs.

STRUCTURAL INTERACTION OF FOOD-DERIVED LIPIDS WITH nsLTPs

Biological Properties of nsLTPs

Non-specific lipid transfer proteins represent a family of small and ubiquitously expressed plant proteins belonging to the prolamin superfamily. nsLTPs are cysteine-rich proteins which are stabilized by four internal disulfide bonds. Two nsLTP subfamilies, 9 kDa nsLTP1 and 7 kDa nsLTP2, are known, both characterized by a globular α-helical structure with a tunnel-like hydrophobic cavity. This cavity makes them suitable for binding and transportation of various lipids (13, 14). Although the physiological function of nsLTPs is contentious, strong evidence has been provided for nsLTPs to function as intra- and extracellular carriers for a broad spectrum of lipids required for membrane biogenesis. Furthermore, nsLTPs are members of the family of pathogenesis-related proteins 14 and are involved in the adaption of plants to abiotic and biotic stress. The role of nsLTPs in plant defense is suggested to result from (1) a direct antimicrobial activity facilitated by permeabilization of cell membranes of phytopathogenic bacteria and fungi and (2) a putative fungal elicitin (nsLTP homolog) receptor-dependent activation of the plant immune system in the presence of lipids (15–18). By contrast, several reports suggest that lipid-binding and antimicrobial properties are spatially separated (19).

Interaction of nsLTPs With Lipids

The majority of studies investigating the structural interaction of lipid ligands with plant nsLTPs has been performed in monocotyledons, e.g., barley, rice, wheat, or maize, to elucidate their physiological function (17). nsLTPs can bind a wide range of ligands, including molecules of organic solvents, certain drugs, acyl derivatives of coenzyme A, sterols, prostaglandin B2 (PGB_2), and likely most importantly aliphatic lipids, comprising glycerophospholipids (PG and PC) and derivatives thereof, including lysophospholipids (lyso-PG and lyso-PC), as well as FAs or FA-dervatives (14, 20). Plant nsLTPs can bind one or two fatty acyl chains by non-cooperative or cooperative binding sites with K_Ds in the low micromolar range: ^{1}H NMR and 2.1-Å crystal structure analysis revealed that TaLTP1.1 from wheat (*Triticum aestivum*) can accommodate either one molecule of 1,2-dimyristoyl-PG or two molecules of 1-myristoyl-2-hydroxy-PC (L-α-myristoyl-PC, lyso-PC, LMPC) in a "head-to-tail" orientation (14, 20). In addition, TaLTP1.1 was crystallized in a complex with PGB_2, an FA derivative originated from C_{20}-unsaturated arachidonic acid. Moreover, studies provided evidence that nsLTPs form the most stable complexes with different C10–C18 chain unsaturated FAs containing one or two double bonds in the *cis* configuration, e.g., linoleic and oleic acid (18). Using pea nsLTP1 it was reported that unsaturated FA, e.g., linoleic (C18:2, all-*cis*-9,12) and linolenic (C18:3, all-*cis*-9,12,15) acids rather than saturated FAs, and lysolipids, e.g., negatively charged LMPG (C14) and L-α-palmitoyl-phosphatidylglycerol (LPPG) (C16), showed strongest interaction with the allergen (21). Notably a tomato LTP was found to bind L-α-palmitoyl-lysophosphatidylcholine (C16). This complex was stable even after thermal treatments with up to 105°C (22).

Structural Modification Induced by Lipid Binding

Of note, nsLTPs lack a marked specificity for ligands which can be attributed to the flexibility of the van der Waals volume of internal hydrophobic cavities sufficient to accommodate either single- or double-chain lipids. The structural variability of the internal cavity has been shown by a more than fourfold increase of the cavity volume after binding of FAs (18). The volume of the cavity of TaLTP1.1 increases from 300 ± 50 to 786 ± 43 Å^3 upon PGB_2-binding (14). Lipid binding to nsLTPs

by the engagement of conserved amino acids at the entrance of the tunnel and subsequent reshaping of the internal hydrophobic cavity is accompanied by a conformational (micro)heterogeneity of the molecules. So far, it is unclear whether the conformational modification affects biological function and allergenicity of the lipid-complexed nsLTPs.

Lipid Binding to nsLTP1 and nsLTP2

Several studies demonstrated the heterogeneity of lipid interaction with nsLTPs from different plant species, between nsLTP isoforms, or members of the nsLTP1 and nsLTP2 subfamilies. However, it seems that the highly conserved amino acids Arg_{44} and Tyr_{79} in nsLTP1 (e.g., from rice) and Phe_{36}, Tyr_{45}, and Tyr_{48} in nsLTP2 at the entrance of the hydrophobic cavity are crucial for lipid "uptake" (18). Hence, these data suggest that all nsLTPs are capable to bind lipids, but the specificity and binding affinity of the ligands cannot be deduced. Furthermore, wheat nsLTP1 (TaLTP1.1) has been shown to bind LMPC (PDB 2BWO) (20), whereas a crystal structure showed wheat nsLTP2 (TaLTP2.1) to interact with LPPG (23). In addition, nsLTP1 has been reported to accommodate either one or two molecules of linear mono- or diacylated lipids, whereas nsLTP2 can bind even planar sterol molecules (19). The binding properties of nsLTPs were attributed to the size of the hydrophobic cavity being more spacious for nsLTP2. Another study showed the cavity of rice nsLTP2, although it is smaller than that of rice nsLTP1, to be flexible enough to accommodate the voluminous sterol molecule (24).

In summary, the structural interaction between lipid ligands and nsLTPs shows the diversity of bound ligands and the heterogeneity of binding modalities. However, the question remains whether the type and mode of lipid binding determines the biological function and whether it affects the allergenic properties of nsLTPs.

INTERACTION OF LIPIDS WITH ALLERGENIC FOOD nsLTPs LIKELY EFFECTS THE STABILITY AND IgE-REACTIVITY OF THE COMPLEXED ALLERGEN

Plant-derived nsLTPs are among the clinically most important food allergens frequently eliciting severe clinical reactions (25, 26). Curiously, nsLTPs are described as allergens in the Mediterranean area rather than in Central and Northern Europe. Actually, more than 35 nsLTPs, almost all belonging to the nsLTP1 subfamily, have been described as food allergens (according the IUIS allergen nomenclature subcommittee) (26). So far, the strong allergenic properties of nsLTPs are attributed to their high resistance to both heat treatment and gastrointestinal enzymes (26). However, up to now only a limited number of studies investigated the interaction between lipids and allergenic nsLTPs and its effects on IgE-binding capacity and allergenic potency.

Peach nsLTP Pru p 3, the prototypic member of the nsLTP family, has been described as a genuine allergen inducing primary sensitization (26). *In vitro* Pru p 3 was able to cross Caco 2 monolayers (27). The transcellular transport of Pru p 3 occurred *via* a lipid raft pathway that did not disturb the integrity of the tight junctions but induced epithelial cell-derived production of the T_H2-promoting cytokines TSLP, IL-33, and IL-25 (27).

The hydrophobic cavity of Pru p 3 displays a remarkable degree of plasticity allowing for the binding of ligands in different orientations (25). From structural analysis, the authors assumed a hypothetical ligand bound to Pru p 3 resembling lauric acid. *In silico* analysis revealed the amino acid residues supposed to participate in lipid binding are conserved in the allergenic peach nsLTP Pru p 3 (21).

Notably linoleic, palmitic, and oleic acids are described as main components of peach oil (28), and linoleic acid has been found as major FA in wheat (29). Recently, substantial binding of palmitic (and linoleic) acid to Pru p 3 rather than to wheat LTP was demonstrated experimentally (29). Surprisingly, lipid binding to wheat LTP was associated with an increased susceptibility to gastrointestinal proteolysis, which was due to a conformational change and the exposure of an additional protease cleavage site (29). Nevertheless, the authors speculated that lipid binding to other nsLTPs may have an opposite effect by increasing the stability and therefore may be capable to contribute to the allergenicity of the molecules/respective allergens. In line with this, Vassilopoulou and co-workers showed a slightly protective effect to the gastric digestion of PC admixed with grape LTP (30). However, the protease-treated nsLTP:lipid complex did not show an increased allergenicity.

Of note, unsaturated FA (oleic, linoleic, and elaidic acid) and short chain saturated lauric acid (C12) were confirmed to bind to Pru p 3 by 1,8-ANS-competition assays (31). Binding of oleic but not stearic acid (saturated C18) increased IgE-reactivity and basophil activation by conformational changes. This effect was less pronounced for allergenic nsLTPs Jug r 3 (English walnut) and Cor a 8 (hazelnut) (32).

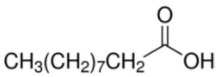
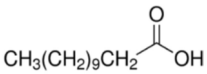
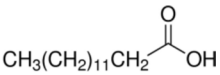
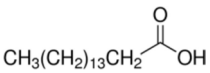
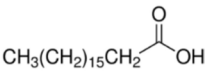
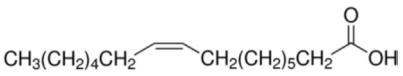
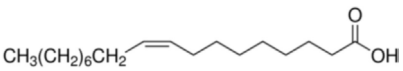
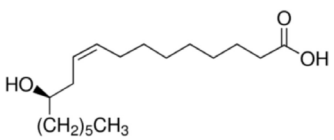
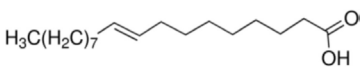
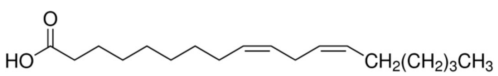
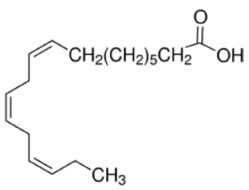
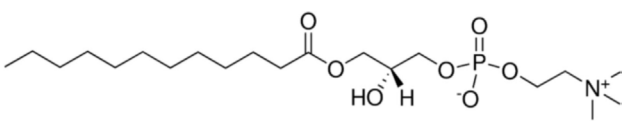
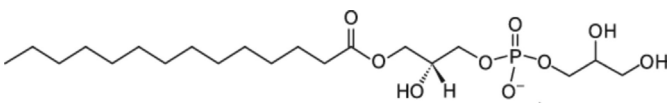
An independent study suggested a derivative of 10-OH-camptothecin potentially bound to the hydrophobic tail of phytosphingosine (C18) as the natural ligand for Pru p 3 (33). Both molecules, identified by ESI-qToF, were co-purified together with natural Pru p 3 (33). *In silico* analysis suggested phytosphingosine to be bound to the hydrophobic cavity of Pru p 3. Here, ligand binding was associated with a slight structural modification of Pru p 3 and a distortion of the CD spectrum of Pru p 3 in the 190–200 nm region (33).

In summary, several FAs, likewise saturated lauric acid and unsaturated oleic acid (31) as well as palmitic acid (29), and phytosphingosine (33) were described as ligands for Pru p 3 (**Table 1**). The conjugation of lipids to nsLTPs may influence the IgE-binding capacity by structural modification of allergens (31). So far, the effect of lipids on the stability of allergens is discussed controversially.

INTERACTION OF LIPIDS WITH ALLERGENIC FOOD nsLTPs AND ITS EFFECT ON THE ALLERGIC SENSITIZATION

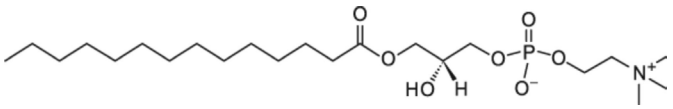
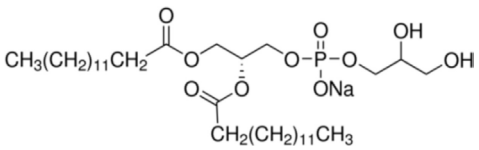
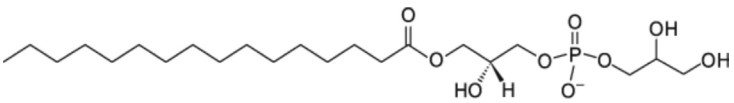
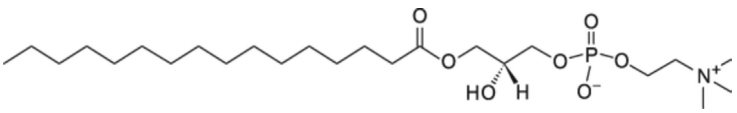
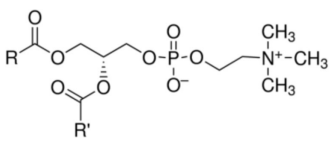
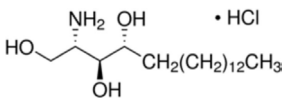
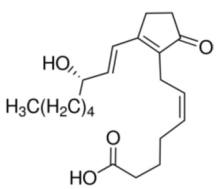
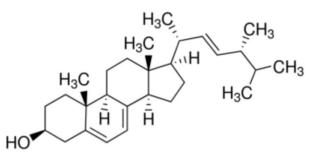
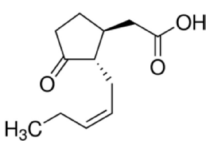
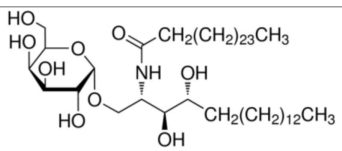
Apart from the effect of lipid binding on the stability and IgE-reactivity of nsLTPs, the interaction with lipids might increase

TABLE 1 | Lipophilic ligands of food-derived nsLTPs.

Fatty acid	Formula	nsLTP	Reference
Decanonic acid [C10]		**Maize LTP**	(14)
Lauric acid [C12]		**Pru p 3** **Lentil LTP** Maize LTP	(29) (14)
Myristic acid [C14]		**Maize LTP** **Rice LTP**	(14)
Palmitic acid [C16]		**Pru p 3** (Tri a 14) Maize LTP Barley LTP	(31) (14) (14)
Stearic acid [C18]		(Pru p 3) (Jug r 3) (Cor a 8) Maize LTP Rice LTP	(31, 32)
Palmitoleic acid [C16:1, cis-9]		(Pea LTP) Maize LTP Rice LTP	(21)
Oleic acid [C18:1, cis-9]		**Prup3** (Jug r 3) (Cor a 8) (Mal d 3) Maize LTP	(31, 32)
Ricinoleic acid [C18:1, cis-9, 12-OH]		**Maize LTP**	(14)
Elaidic acid [C18:1, trans-9]		**Pru p 3**	(31)
Linoleic acid [C18:2, cis-9,12]		**Prup 3** **Pea LTP** **Pru p 3**	(21, 29, 31)
Linoleinic acid [C18:3 cis-9,12,15]		**Pea LTP** (Tri a 14) (Maize LTP)	(21) (29) (14)
(Phospho) Lipids			
LLPC [C12]		Barley LTP	(21)
LMPG [C14]		Pea LTP	(21)

(*Continued*)

TABLE 1 | Continued

Fatty acid	Formula	nsLTP	Reference
(Phospho) Lipids			
LMPC [C14]		**Wheat LTP1** **Pea LTP**	(20) (14)
DMPG [C14/C14]		**Wheat LTP1**	(20)
LPPG [C16]		**Wheat LTP2** **Pea LTP**	(23) (21)
LPPC [C16]		**Tomato LTP** **Pea LTP**	(22) (21)
Phosphatidylcholin		Grape LTP (admixed) **Maize LTP > wheat LTP**	(30) (34)
Others			
Phytosphingosine [C18]		**Pru p 3**	(33)
Prostaglandin B2		**Wheat LTP1**	(14)
(Ergo)sterol		**LTP2** **Rice LTP2**	(19, 24) (24)
Jasmonic acid		Pea LTP	(21)
α-GalCer		CD1d ligand (control)	(35)

Chemical structure of lipids and nsLTPs reported to bind the respective lipids are indicated. nsLTPs with strongest lipid binding capacity are indicated in bold, allergenic nsLTPs are indicated according to nomenclature suggested by the IUIS allergen nomenclature subcommittee.

LLPC, 1-lauroyl-2-hydroxy-sn-glycero-3-phosphocholine; LMPG, 1-myristoyl-2-hydroxy-sn-glycero-3-phospho-(1′-rac-glycerol); LMPC, 1-myristoyl-2-hydroxy-sn-glycero-3-phosphocholine; DMPG, 1,2-dimyristoyl-sn-glycero-3-phospho-(1′-rac-glycerol); LPPG, 1-palmitoyl-2-hydroxy-sn-glycero-3-phospho-(1′-rac-glycerol); LPPC, 1-myristoyl-2-hydroxy-sn-glycero-3-phosphocholine.

nsLTPs, non-specific lipid-transfer proteins; LPPG, L-α-palmitoyl-phosphatidylglycerol; LPPC, L-α-palmitoyl-lysophosphatidylcholine.

immunogenicity of the antigen. Although lipid binding is a common feature of many allergens (6), studies addressing the molecular mechanism by which the innate and allergen-specific adaptive immune responses are triggered are limited.

Recently, it has been reported that an olive pollen-derived lipid fraction was capable to upregulate CD1d (a class I MHC-like molecule) expression on human macrophages (Mϕ) and moDCs, and to activate invariant natural killer T (iNKT) cells (36, 37). Although an allergen-specific response was not investigated, the authors suggested lipid binding to the allergen to have an influence on the allergen-specific immune response. Experimental evidence was provided that binding of a natural lipid ligand to Pru p 3 provokes the allergic sensitization process by involvement of CD1d-mediated activation of iNKT cells (38). The suggested ligand of Pru p 3, 10-OH-camptothecin-phytosphingosine, was presented *via* CD1d on antigen-presenting cells to interact with human (and mouse) iNKTs and was shown to act as an adjuvant to promote IgE-sensitization to Pru p 3 (**Figure 1**). Notably, α-GalCer, which is well known to induce type I iNKT cell activation, comprises a phytosphingosine, which is an important component for binding to CD1d (35, 39). Interestingly, the complex, but also the lipid ligand alone, induced maturation of human moDCs and proliferation of PBMCs, and its component phytosphingosine was capable to activate NF-kB signaling in the human Mϕ cell line THP-1 (35). By contrast, another study showed the anti-inflammatory

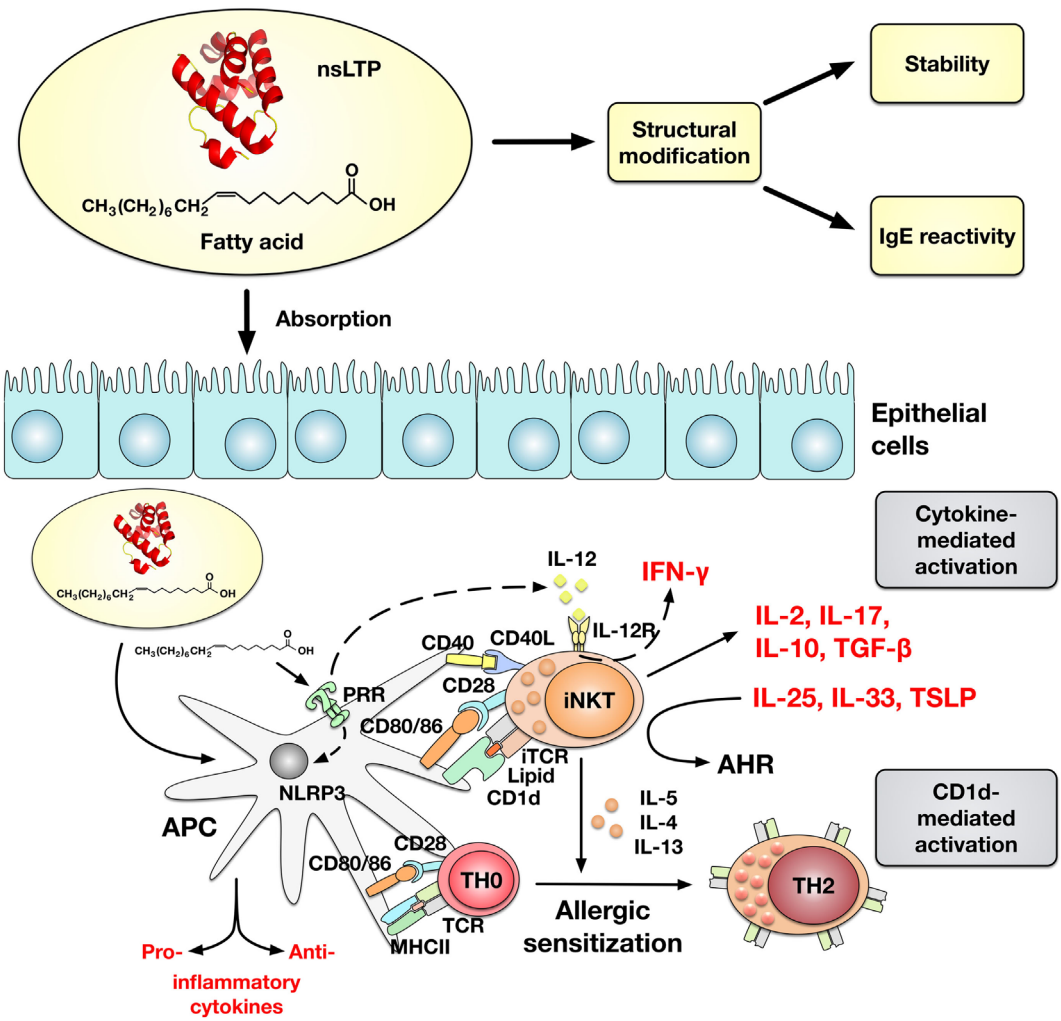


FIGURE 1 | Impact of lipid binding to non-specific lipid-transfer proteins (nsLTPs) on the IgE-reactivity and allergic sensitization. Lipid binding can affect gastrointestinal stability and IgE-reactivity of nsLTPs. Whether the altered IgE-reactivity upon lipid binding is accompanied by structural modification of the protein remains to be elucidated. Moreover, lipids can induce a cytokine- or CD1d-mediated activation of invariant natural killer T (iNKT) cells. Microbial lipids are sensed by pathogen recognition receptors (PRRs) on APCs (DC and Mϕ) and induce an IL-12-mediated activation of iNKT cells which is accompanied by INF-γ secretion. Other lipid ligands which are administrated to APCs in a complex with nsLTPs are internalized, loaded on CD1d, and presented to the TCR (Vα24/Jα18$^+$ in humans) on iNKT cells. The CD1d-restricted activation of iNKT cells by nsLTP–lipid complexes is suggested to promote the allergic sensitization by inducing T_H2-cytokine secretion. In line with this, iNKT cells can promote airway hyperreactivity (AHR) in the presence of IL-25, IL-33, and TSLP. However, activated iNKT cells can display a T_H1- (INF-γ) and T_H2- (IL-5, IL-4, and IL-13) like plasticity and lipids can either promote or attenuate NLRP3 inflammasome activity and induce both pro- or anti-inflammatory cytokines.

activity of phytosphingosine by inhibiting the NF-kB pathway (40). Remarkably, epicutaneous sensitization of BALB/c mice by the Pru p 3-ligand complex induced higher levels of Pru p 3-specific IgE antibodies and enhanced basophil activation in comparison to Pru p 3 alone (38). The authors suggested (1) Pru p 3 to act as carrier and to mimic endogenous saposins which are involved in the loading of lipids to CD1d and (2) the intrinsic adjuvant activity of the accompanying lipid cargo could be a general and essential feature of the mechanism underlying the phenomenon of nsLTP-mediated allergy. Of note, a modification of the lipid cargo phytosphingosine chain can manipulate the iNKT cells to produce different amounts of either IL-4 or IFN-γ polarizing immune response toward either T_H1 or T_H2 (35).

In the study by Tordesillas and co-workers (38), the adjuvant effect of lipid alone in regard to its effects *in vivo*, its properties to modulate cytokine secretion in PBMCs, and the CD1d-mediated lipid presentation without Pru p 3 as carrier were not addressed. Although, the interaction of lipids with nsLTPs likely affects the development of an allergic inflammation, it remains to be elucidated whether lipid binding into the cavity of nsLTPs is required to exert all immune-modulating properties. So far, the effect of other described lipid ligands on the allergen-specific immune response to nsLTPs is poorly characterized and it remains unknown whether FA ligands of Pru p 3 have immunological properties to activate iNKT cells.

DISCUSSION

Non-specific lipid-transfer proteins can bind a broad variety of lipids due to versatile binding abilities of the proteins. Whether the lack of binding specificity can be explained with a structural flexibility of the binding cavity which accounts for the variable lipid-binding modalities between different nsLTPs and isoforms is controversially discussed (41). Moreover, the lipid binding capacity of nsLTPs depends on the chemical nature of the ligand and key amino acids at the entrance of the hydrophobic cavity. The interaction of certain lipids with nsLTPs has been suggested to affect both the IgE-reactivity and immunogenicity of the respective allergens. The hypothesis is deduced from reports that lipid binding to nsLTPs can slightly alter protein structure and therefore also conformational IgE-binding sites affecting the stability and IgE-binding properties of nsLTPs. However, whether lipid binding to nsLTPs increases the intrinsic immunogenicity, by inducing biologically relevant structural changes in the resulting nsLPT:lipid complex and/or by activation the innate immune system is not fully clarified at the moment.

In general, precise analysis of the nsLTP–lipid complex is required to discriminate whether lipids are bound to the cavity of nsLTPs or are simply attached to the surface of the protein. Moreover, for the assessment of lipid binding properties of nsLTPs any natural, pre-loaded lipids need to be excluded. This is of particular importance since the purification of recombinant nsLTPs can be associated with a co-purification and binding of lipids derived from the natural source or the applied heterologous expression system (25). Finally, it should be taken into account that the composition of lipids and free sterols changes due to development and ripening of fruits (42).

Whereas the mode of interaction between lipids and nsLTPs is well investigated, the effects of lipid binding on the allergic sensitization and the molecular mechanisms in effector cells are largely unknown. In line with this, it could also be speculated that nsLTP interaction with lipids will protect from gastrointestinal digestion and promote the absorption of intact nsLTPs into the intestinal tissue and blood stream. Although not experimentally proven, the interaction of nsLTPs with the lipid bilayer of the cell membrane might mediate a facilitated endocytosis and subsequent antigen processing and presentation.

The interaction or co-exposure of lipids together with nsLTPs is an attractive model to further explain the pathomechanism of nsLTP-mediated allergies, but also other hypersensitivity reactions triggered by allergens with lipid-binding properties. Here, the suggested function of nsLTPs as lipid carriers, e.g., to target lipid adjuvants and antigen simultaneously to APCs, needs to be further investigated. So far, lipid binding to nsLTPs has been shown to result in CD1d-restricted activation of iNKT cells promoting allergic sensitization (**Figure 1**). However, additional mechanisms, likewise lipids engaging pathogen recognition receptors (PRRs) and lipids influencing the absorption of allergens through the epithelial barrier, might be involved to modulate the immunogenicity of nsLTP:lipid complexes (43). Future research needs to consider that nsLTP-associated lipids potentially can function as danger signals to activate PRRs and can influence the NLRP3 inflammasome in APCs. Specifically, saturated FAs have been shown to promote inflammation and polyunsaturated FAs have been shown to impede inflammasome activity (44). Whether FA delivered to APCs by nsLTP cargo proteins are be capable to modulate inflammasome activity has not been investigated. Taking into account the diversity of lipid ligands, it could be speculated that both pro- or anti-inflammatory immune responses and T_H1- or T_H2-polarized antigen-specific immune responses could be induced, respectively. In fact, lipids possess intrinsic adjuvant activity and likely can modulate the allergenicity of proteins not only by altering protein structure and stability upon binding (29) but also by direct interaction of bound and unbound lipids with immune cells. Free FA can modulate proliferation and cytokine secretion of T cells differentially, depending on their saturation grade and the stimuli used (43).

The reported studies describing lipid binding to nsLTPs have used different experimental approaches to tackle the complex task of characterizing lipid binding to nsLTPs: (1) *in silico* experiments by molecular modeling have been applied to predict interactions between different lipid ligands and the binding cavity of the respective nsLTP, (2) purified nsLTPs have been exposed to different lipids *in vitro* to determine the binding capacity of the respective lipids to the tested nsLTP and finally, and (3) lipids ligands were isolated from natural Pru p 3 in order to identify the lipids naturally bound to the allergen.

While *in vitro* binding experiments have shown that, in accordance with their function as nsLTPs, the different nsLTPs tested can bind a variety of different lipids, unexpectedly the lipid isolation experiments using natural purified Pru p 3 reported a single, major lipid ligand to be bound to the cavity of Pru p 3. Here, future research should aim to systematically characterize ligand binding to both allergenic- and non-allergenic LTPs. It

is tempting to speculate that lipid binding and/or the activation of the innate immune system lipids contributes to the higher allergenicity of certain nsLTPs.

AUTHOR CONTRIBUTIONS

Both authors contributed equally to the manuscript.

ACKNOWLEDGMENTS

The authors thank Andrea Wangorsch for support with figure arrangement and Stefan Vieths for revision of the manuscript.

FUNDING

This work was funded by the budget of the Paul-Ehrlich-Institut.

REFERENCES

1. Sicherer SH, Sampson HA. Food allergy: a review and update on epidemiology, pathogenesis, diagnosis, prevention, and management. *J Allergy Clin Immunol* (2018) 141:41–58. doi:10.1016/j.jaci.2017.11.003
2. Gupta R, Sheikh A, Strachan DP, Anderson HR. Burden of allergic disease in the UK: secondary analyses of national databases. *Clin Exp Allergy* (2004) 34:520–6. doi:10.1111/j.1365-2222.2004.1935.x
3. Scheurer S, Toda M, Vieths S. What makes an allergen? *Clin Exp Allergy* (2015) 45:1150–61. doi:10.1111/cea.12571
4. Gómez-Casado C, Díaz-Perales A. Allergen-associated immunomodulators: modifying allergy outcome. *Arch Immunol Ther Exp (Warsz)* (2016) 64:339–47. doi:10.1007/s00005-016-0401-2
5. Thomas WR. Allergen ligands in the initiation of allergic sensitization. *Curr Allergy Asthma Rep* (2014) 14:432. doi:10.1007/s11882-014-0432-x
6. Bublin M, Eiwegger T, Breiteneder H. Do lipids influence the allergic sensitization process? *J Allergy Clin Immunol* (2014) 134:521–9. doi:10.1016/j.jaci.2014.04.015
7. Dearman RJ, Alcocer MJC, Kimber I. Influence of plant lipids on immune responses in mice to the major Brazil nut allergen Ber e 1. *Clin Exp Allergy* (2007) 37:582–91. doi:10.1111/j.1365-2222.2007.02689.x
8. Mirotti L, Florsheim E, Rundqvist L, Larsson G, Spinozzi F, Leite-de-Moraes M, et al. Lipids are required for the development of Brazil nut allergy: the role of mouse and human iNKT cells. *Allergy* (2013) 68:74–83. doi:10.1111/all.12057
9. Sancho AI, Wangorsch A, Jensen BM, Watson A, Alexeev Y, Johnson PE, et al. Responsiveness of the major birch allergen Bet v 1 scaffold to the gastric environment: impact on structure and allergenic activity. *Mol Nutr Food Res* (2011) 55:1690–9. doi:10.1002/mnfr.201100025
10. Angelina A, Sirvent S, Palladino C, Vereda A, Cuesta-Herranz J, Eiwegger T, et al. The lipid interaction capacity of Sin a 2 and Ara h 1, major mustard and peanut allergens of the cupin superfamily, endorses allergenicity. *Allergy* (2016) 71:1284–94. doi:10.1111/all.12887
11. Chung S-Y, Mattison CP, Reed S, Wasserman RL, Desormeaux WA. Treatment with oleic acid reduces IgE binding to peanut and cashew allergens. *Food Chem* (2015) 180:295–300. doi:10.1016/j.foodchem.2015.02.056
12. Palladino C, Narzt MS, Bublin M, Schreiner M, Humeniuk P, Gschwandtner M, et al. Peanut lipids display potential adjuvanticity by triggering a pro-inflammatory response in human keratinocytes. *Allergy* (2018) 348:977. doi:10.1111/all.13475
13. Kader J-C. Lipid-transfer proteins in plants. *Annu Rev Plant Physiol Plant Mol Biol* (1996) 47:627–54. doi:10.1146/annurev.arplant.47.1.627
14. Salminen TA, Blomqvist K, Edqvist J. Lipid transfer proteins: classification, nomenclature, structure, and function. *Planta* (2016) 244:971–97. doi:10.1007/s00425-016-2585-4
15. Buhot N, Douliez JP, Jacquemard A, Marion D, Tran V, Maume BF, et al. A lipid transfer protein binds to a receptor involved in the control of plant defence responses. *FEBS Lett* (2001) 509:27–30. doi:10.1016/S0014-5793(01)03116-7
16. Buhot N, Gomès E, Milat M-L, Ponchet M, Marion D, Lequeu J, et al. Modulation of the biological activity of a tobacco LTP1 by lipid complexation. *Mol Biol Cell* (2004) 15:5047–52. doi:10.1091/mbc.e04-07-0575
17. Yeats TH, Rose JKC. The biochemistry and biology of extracellular plant lipid-transfer proteins (LTPs). *Protein Sci* (2008) 17:191–8. doi:10.1110/ps.073300108
18. Finkina EI, Melnikova DN, Bogdanov IV, Ovchinnikova TV. Lipid transfer proteins as components of the plant innate immune system: structure, functions, and applications. *Acta Naturae* (2016) 8:47–61.
19. Stotz HU, Waller F, Wang K. Innate immunity in plants: the role of antimicrobial peptides. In: Hiemstra PS, Zaat SAJ, editors. *Antimicrobial Peptides and Innate Immunity*. Basel: Springer (2013). p. 29–51.
20. Charvolin D, Douliez JP, Marion D, Cohen-Addad C, Pebay-Peyroula E. The crystal structure of a wheat nonspecific lipid transfer protein (ns-LTP1) complexed with two molecules of phospholipid at 2.1 A resolution. *Eur J Biochem* (1999) 264:562–8. doi:10.1046/j.1432-1327.1999.00667.x
21. Bogdanov IV, Shenkarev ZO, Finkina EI, Melnikova DN, Rumynskiy EI, Arseniev AS, et al. A novel lipid transfer protein from the pea *Pisum sativum*: isolation, recombinant expression, solution structure, antifungal activity, lipid binding, and allergenic properties. *BMC Plant Biol* (2016) 16:107. doi:10.1186/s12870-016-0792-6
22. Volpicella M, Leoni C, Fanizza I, Rinalducci S, Placido A, Ceci LR. Expression and characterization of a new isoform of the 9 kDa allergenic lipid transfer protein from tomato (variety San Marzano). *Plant Physiol Biochem* (2015) 96:64–71. doi:10.1016/j.plaphy.2015.07.019
23. Hoh F, Pons J-L, Gautier M-F, de Lamotte F, Dumas C. Structure of a liganded type 2 non-specific lipid-transfer protein from wheat and the molecular basis of lipid binding. *Acta Crystallogr D Biol Crystallogr* (2005) 61:397–406. doi:10.1107/S0907444905000417
24. Cheng C-S, Samuel D, Liu Y-J, Shyu J-C, Lai S-M, Lin K-F, et al. Binding mechanism of nonspecific lipid transfer proteins and their role in plant defense. *Biochemistry* (2004) 43:13628–36. doi:10.1021/bi048873j
25. Pasquato N, Berni R, Folli C, Folloni S, Cianci M, Pantano S, et al. Crystal structure of peach Pru p 3, the prototypic member of the family of plant non-specific lipid transfer protein pan-allergens. *J Mol Biol* (2006) 356:684–94. doi:10.1016/j.jmb.2005.11.063
26. Petersen A, Scheurer S, Jörg K-T. Molecular allergy diagnostics. In: Kleine-Tebbe J, Jakob T, editors. *Stable Plant Food Allergens I: Lipid-Transfer-Proteins*. Switzerland: Springer International Publishing (2017). doi:10.1007/978-3-319-42499-6
27. Tordesillas L, Gómez-Casado C, Garrido-Arandia M, Murua-García A, Palacín A, Varela J, et al. Transport of Pru p 3 across gastrointestinal epithelium – an essential step towards the induction of food allergy? *Clin Exp Allergy* (2013) 43:1374–83. doi:10.1111/cea.12202
28. Kikalishvili BI, Zurabashvili DZ, Turabelidze DG, Shanidze LA, Nikolaïshvili MN. [The fatty acid composition of peach oil and its biological activity]. *Georgian Med News* (2013) 218:82–5.
29. Abdullah SU, Alexeev Y, Johnson PE, Rigby NM, Mackie AR, Dhaliwal B, et al. Ligand binding to an allergenic lipid transfer protein enhances conformational flexibility resulting in an increase in susceptibility to gastroduodenal proteolysis. *Sci Rep* (2016) 6:30279. doi:10.1038/srep30279
30. Vassilopoulou E, Rigby N, Moreno FJ, Zuidmeer L, Akkerdaas J, Tassios I, et al. Effect of in vitro gastric and duodenal digestion on the allergenicity of grape lipid transfer protein. *J Allergy ClinImmunol* (2006) 118:473–80. doi:10.1016/j.jaci.2006.04.057
31. Dubiela P, Aina R, Polak D, Geiselhart S, Humeniuk P, Bohle B, et al. Enhanced Pru p 3 IgE-binding activity by selective free fatty acid-interaction. *J Allergy Clin Immunol* (2017) 140:1728–31.e10. doi:10.1016/j.jaci.2017.06.016
32. Dubiela P, Aina R, Humenuik P, Peifer S, Bublin M, Cantini F, et al. Impact of free fatty acids binding to nsLTP on their tertiary structure and allergenic activity. *Food Allergy Anaphylaxis Meeting*. Rome, Italy (2016).

33. Cubells-Baeza N, Gómez-Casado C, Tordesillas L, Ramírez-Castillejo C, Garrido-Arandia M, González-Melendi P, et al. Identification of the ligand of Pru p 3, a peach LTP. *Plant Mol Biol* (2017) 94:33–44. doi:10.1007/s11103-017-0590-z
34. Guerbette F, Grosbois M, Jolliot-Croquin A, Kader JC, Zachowski A. Comparison of lipid binding and transfer properties of two lipid transfer proteins from plants. *Biochemistry* (1999) 38:14131–7. doi:10.1021/bi990952l
35. Hung J-T, Huang J-R, Yu AL. Tailored design of NKT-stimulatory glycolipids for polarization of immune responses. *J Biomed Sci* (2017) 24:22. doi:10.1186/s12929-017-0325-0
36. Abós-Gracia B, López-Relaño J, Revilla A, Castro L, Villalba M, Martín Adrados B, et al. Human invariant natural killer T cells respond to antigen-presenting cells exposed to lipids from *Olea europaea* pollen. *Int Arch Allergy Immunol* (2017) 173:12–22. doi:10.1159/000467394
37. Abós-Gracia B, del Moral MG, López-Relaño J, Viana-Huete V, Castro L, Villalba M, et al. *Olea europaea* pollen lipids activate invariant natural killer T cells by upregulating CD1d expression on dendritic cells. *J Allergy Clin Immunol* (2013) 131:1393–9.e5. doi:10.1016/j.jaci.2012.11.014
38. Tordesillas L, Cubells-Baeza N, Gómez-Casado C, Berin C, Esteban V, Barcik W, et al. Mechanisms underlying induction of allergic sensitization by Pru p 3. *Clin Exp Allergy* (2017) 47:1398–408. doi:10.1111/cea.12962
39. Dangerfield EM, Cheng JMH, Knight DA, Weinkove R, Dunbar PR, Hermans IF, et al. Species-specific activity of glycolipid ligands for invariant NKT cells. *Chembiochem* (2012) 13:1349–56. doi:10.1002/cbic.201200095
40. Kim B-H, Lee JM, Jung Y-G, Kim S, Kim T-Y. Phytosphingosine derivatives ameliorate skin inflammation by inhibiting NF-κB and JAK/STAT signaling in keratinocytes and mice. *J Invest Dermatol* (2014) 134:1023–32. doi:10.1038/jid.2013.453
41. del Moral MG, Martínez-Naves E. The role of lipids in development of allergic responses. *Immune Netw* (2017) 17:133–43. doi:10.4110/in.2017.17.3.133
42. Izzo R, Scartazza A, Masia A, Gallelschi L, Quartacci MF, Navari-Izzo F. Lipid evolution during development and ripening of peach fruits. *Phytochemistry* (1995) 39:1329–34. doi:10.1016/0031-9422(95)00189-E
43. de Jong AJ, Kloppenburg M, Toes REM, Ioan-Facsinay A. Fatty acids, lipid mediators, and T-cell function. *Front Immunol* (2014) 5:483. doi:10.3389/fimmu.2014.00483
44. Ralston JC, Lyons CL, Kennedy EB, Kirwan AM, Roche HM. Fatty acids and NLRP3 inflammasome-mediated inflammation in metabolic tissues. *Annu Rev Nutr* (2017) 37:77–102. doi:10.1146/annurev-nutr-071816-064836

Conflict of Interest Statement: The authors declare that the submitted work was not carried out in the presence of any personal, professional, or financial relationships that could potentially be construed as a conflict of interest.

frontiers in Immunology

ORIGINAL RESEARCH
published: 14 August 2018
doi: 10.3389/fimmu.2018.01888

Bradyrhizobium Lipid A: Immunological Properties and Molecular Basis of Its Binding to the Myeloid Differentiation Protein-2/ Toll-Like Receptor 4 Complex

Luigi Lembo-Fazio[1†], Jean-Marc Billod[2†], Flaviana Di Lorenzo[3†], Ida Paciello[1], Mateusz Pallach[3], Sara Vaz-Francisco[4], Aurora Holgado[5,6], Rudi Beyaert[5,6], Manuel Fresno[4], Atsushi Shimoyama[7], Rosa Lanzetta[3], Koichi Fukase[7], Djamel Gully[8], Eric Giraud[8], Sonsoles Martín-Santamaría[2], Maria-Lina Bernardini[1,9]* and Alba Silipo[3]**

[1] Dipartimento di Biologia e Biotecnologie "C. Darwin", Sapienza-Università di Roma, Rome, Italy, [2] Department of Structural and Chemical Biology, Centro de Investigaciones Biológicas, CIB-CSIC, Madrid, Spain, [3] Dipartimento di Scienze Chimiche, Complesso Universitario Monte Sant'Angelo, Università di Napoli Federico II, Naples, Italy, [4] Diomune SL, Parque Científico de Madrid, Madrid, Spain, [5] Center for Inflammation Research, Unit of Molecular Signal Transduction in Inflammation, VIB, Ghent, Belgium, [6] Department of Biomedical Molecular Biology, Ghent University, Ghent, Belgium, [7] Department of Chemistry, Graduate School of Science, Osaka University, Osaka, Japan, [8] IRD, Laboratoire des Symbioses Tropicales et Méditerranéennes (LSTM), UMR IRD/SupAgro/INRA/UM2/CIRAD, TA-A82/J – Campus de Baillarguet, Montpellier, France, [9] Istituto Pasteur Italia – Fondazione Cenci Bolognetti, Sapienza-Università di Roma, Rome, Italy

Lipopolysaccharides (LPS) are potent activator of the innate immune response through the binding to the myeloid differentiation protein-2 (MD-2)/toll-like receptor 4 (TLR4) receptor complexes. Although a variety of LPSs have been characterized so far, a detailed molecular description of the structure–activity relationship of the lipid A part has yet to be clarified. Photosynthetic *Bradyrhizobium* strains, symbiont of *Aeschynomene* legumes, express distinctive LPSs bearing very long-chain fatty acids with a hopanoid moiety covalently linked to the lipid A region. Here, we investigated the immunological properties of LPSs isolated from *Bradyrhizobium* strains on both murine and human immune systems. We found that they exhibit a weak agonistic activity and, more interestingly, a potent inhibitory effect on MD-2/TLR4 activation exerted by toxic enterobacterial LPSs. By applying computational modeling techniques, we also furnished a plausible explanation for the *Bradyrhizobium* LPS inhibitory activity at atomic level, revealing that its uncommon lipid A chemical features could impair the proper formation of the receptorial complex, and/or has a destabilizing effect on the pre-assembled complex itself.

Keywords: lipopolysaccharide, innate immunity, inflammatory cytokines, myeloid differentiation protein-2/toll-like receptor 4, *Bradyrhizobium* lipid A, molecular modeling

OPEN ACCESS

Edited by:
Katarzyna Anna Duda, Forschungszentrum Borstel (LG), Germany

Reviewed by:
Achille Broggi, Harvard University, United States
Anna Swierzko, Institute for Medical Biology (PAN), Poland

***Correspondence:**
Sonsoles Martín-Santamaría
smsantamaria@cib.csic.es;
Maria-Lina Bernardini
maria.bernardini@uniroma1.it;
Alba Silipo
silipo@unina.it

[†]These authors have contributed equally to this work.

Specialty section:
This article was submitted to Molecular Innate Immunity, a section of the journal Frontiers in Immunology

Received: *16 April 2018*
Accepted: *31 July 2018*
Published: *14 August 2018*

Citation:
Lembo-Fazio L, Billod J-M, Di Lorenzo F, Paciello I, Pallach M, Vaz-Francisco S, Holgado A, Beyaert R, Fresno M, Shimoyama A, Lanzetta R, Fukase K, Gully D, Giraud E, Martín-Santamaría S, Bernardini M-L and Silipo A (2018) Bradyrhizobium Lipid A: Immunological Properties and Molecular Basis of Its Binding to the Myeloid Differentiation Protein-2/ Toll-Like Receptor 4 Complex. Front. Immunol. 9:1888. doi: 10.3389/fimmu.2018.01888

INTRODUCTION

Lipopolysaccharides (LPSs) are amphiphilic molecules covering the outer membrane of most Gram-negative bacteria. They are widely known to be involved in the elicitation of immune responses in eukaryotic organisms (1). Structurally, LPSs, in their smooth-form (S-LPS), are tripartite

macromolecules built up of a polysaccharide moiety, termed O-antigen, and a core oligosaccharide region covalently linked to a glycolipid domain termed lipid A; the latter is the most conserved part and is responsible for the immunopotency exerted by LPSs isolated from pathogenic Gram-negative bacteria (2). In mammals, the lipid A component of the LPS is the primary immunostimulatory moiety of Gram-negative bacteria and acts as strong stimulator of the innate immunity. The endotoxic properties of the lipid A, namely its capacity to activate the host innate immune response, are strongly influenced by its primary structure. An overacting immune response, due to an uncontrolled and massive circulation of toxic LPS, can result in severe symptoms of sepsis and, in the worst case, septic shock and multi-organ failure. Interestingly, lipid A displaying moderate to low agonist activity can operate as antagonist reducing or, in a dose-dependent manner, completely inhibiting a lipid A's driven immune activation (1, 3, 4). Lipid A binds the receptor complex made up of toll-like receptor 4 (TLR4) and myeloid differentiation protein-2 (MD-2) on the plasma membrane of immune cells thus activating downstream signaling pathways leading to a rapid release of inflammatory cytokines (1, 5). The highest known immunostimulatory action on human cells is exerted by the *bis*-phosphorylated hexa-acylated lipid A from *Escherichia coli*, characterized by an asymmetric distribution of the acyl chains on the sugar backbone (**Figure 1**). Structural features such as the nature, number, and distribution of the fatty acid (FA) chains as well as the occurrence of phosphate units, greatly regulate its immunopotency; variation in the acylation or phosphorylation pattern corresponds to a decrease in the immunostimulatory (agonist) activity of the lipid A. One of the first known antagonistic lipid A species acting on the human MD-2/TLR4 complex is a tetra-acylated partial structure termed lipid IV_A, a biosynthetic precursor of *E. coli* lipid A devoid of the secondary acyl moieties (6). The X-ray crystallographic structure of human MD-2/TLR4 with *E. coli* hexa-acyl LPS (7) provided the molecular basis of lipid A recognition by the MD-2/TLR4 receptor complex. Briefly, five of the six FA chains of *E. coli* LPS are buried inside the lipophilic pocket of the MD-2 protein whereas the sixth acyl chain is partially extruded, lying on the surface and interacting with the partner TLR4 (dubbed TLR4*). This interaction is the basis for receptor complex dimerization, promoting the intracellular juxtaposition of the TIR domains and leading to signal transduction culminating in the elicitation of the inflammatory process. The X-ray crystallographic structure of the tetra-acylated lipid IV_A in complex with human MD-2/TLR4 receptor demonstrated that all four acyl chains are sitting inside the MD-2 binding pocket in a fashion that does not allow dimerization and subsequent activation (1, 8).

Noticeable efforts have been made so far to identify natural or *ad hoc* synthesized lipid A significantly different from enterobacterial counterparts and able to interfere or modulate immune/inflammatory responses mediated by toxic lipid A, such as lipid IV_A and Eritoran (1, 9). These lipid A variants are the most studied ligands of MD-2/TLR4, which is considered a molecular target related to several inflammatory pathologies but also to "modern-day" diseases, including allergies, asthma, and autoimmune disorders (10). However, the natural repertoire of LPSs is undeniably much more diverse, thus opening the possibility to find natural compounds to active as TLR4 immunomodulators or alternatively, to synthesize bio-inspired lipid A derivatives in a perspective of therapy-related drug design.

In this context, rhizobial lipid A remarkably differs from enterobacterial analogs in terms of acylation and phosphorylation pattern as well as in the sugar backbone. Previous studies reported indeed the weak endotoxic activity of rhizobial lipid A as well as its inhibitory properties toward the toxic effects of enterobacterial LPS (11–14), with the single exception of *S. meliloti* (15). Rhizobia are Gram-negative bacteria able to establish symbiotic relationship with legumes and to reduce atmospheric nitrogen into ammonium, thus providing nitrogen nutrition to the host plants (16, 17). Rhizobia belonging to the *Bradyrhizobium* genus are the most commonly found symbiont for most legume species in habitats worldwide and constitute the most commonly used inoculants for cultivated plants of first agronomic importance (as soybean, peanut, and cowpea) (18–20). *Bradyrhizobium* lipid A (21–24) (**Figure 1**), included the strains ORS278 and ORS285 here used (25), comprises a mixture of species differing by the number, length, and nature of the acyl chains characterized by (i) a pentasaccharide sugar backbone; (ii) the occurrence of very long-chain fatty acids (VLCFA), which have been demonstrated to be pivotal in the bacterium adaptation to intracellular life (26–29); and (iii) a hopanoid moiety covalently linked to the VLCFA, present in a non-stoichiometric fashion.

The observation of expressing the unusual lipid A structure of Bradyrhizobia strains prompted us to evaluate the impact of *Bradyrhizobium* LPS/lipid A on the innate immune system. The results reported herein revealed an extremely low capability to elicit an immune response when tested on both murine and human cells. More intriguingly, a strong inhibitory activity toward the potent agonist *E. coli* LPS was observed. In order to shed light on this behavior, we also investigated the molecular basis of the mechanism of *Bradyrhizobium* LPS/lipid A binding to the human MD-2/TLR4 complex by computational approach, with the aim to provide a molecular model explaining the antagonist properties of *Bradyrhizobium* lipid A.

With this work we meant to complement and expand previous studies highlighting the potential of rhizobia LPS as a promising natural source of MD-2/TLR4 immunomodulatory compounds, that can be of inspiration for development of vaccine adjuvants and/or endotoxin-based therapeutics as an alternative approach in the treatment of inflammatory disorders.

MATERIALS AND METHODS

HEK 293 hTLR4/CD14/MD2 Cell Culture, Transfection, and Stimulation

HEK293 cell line, stably transfected with human TLR4/MD2-CD14 (InvivoGen) was seeded into 96-well plate at the concentration of 1×10^5 cells/mL. 48 h after seeding the cells were transiently transfected through PolyFect Transfection Reagent (Qiagen) with a reaction mix containing 150 ng of Firefly luciferase reporter constructs, pGL3.ELAM.tk [harboring nuclear factor kappa B (NF-κB) promoter sequences], and 15 ng of *Renilla* luciferase reporter

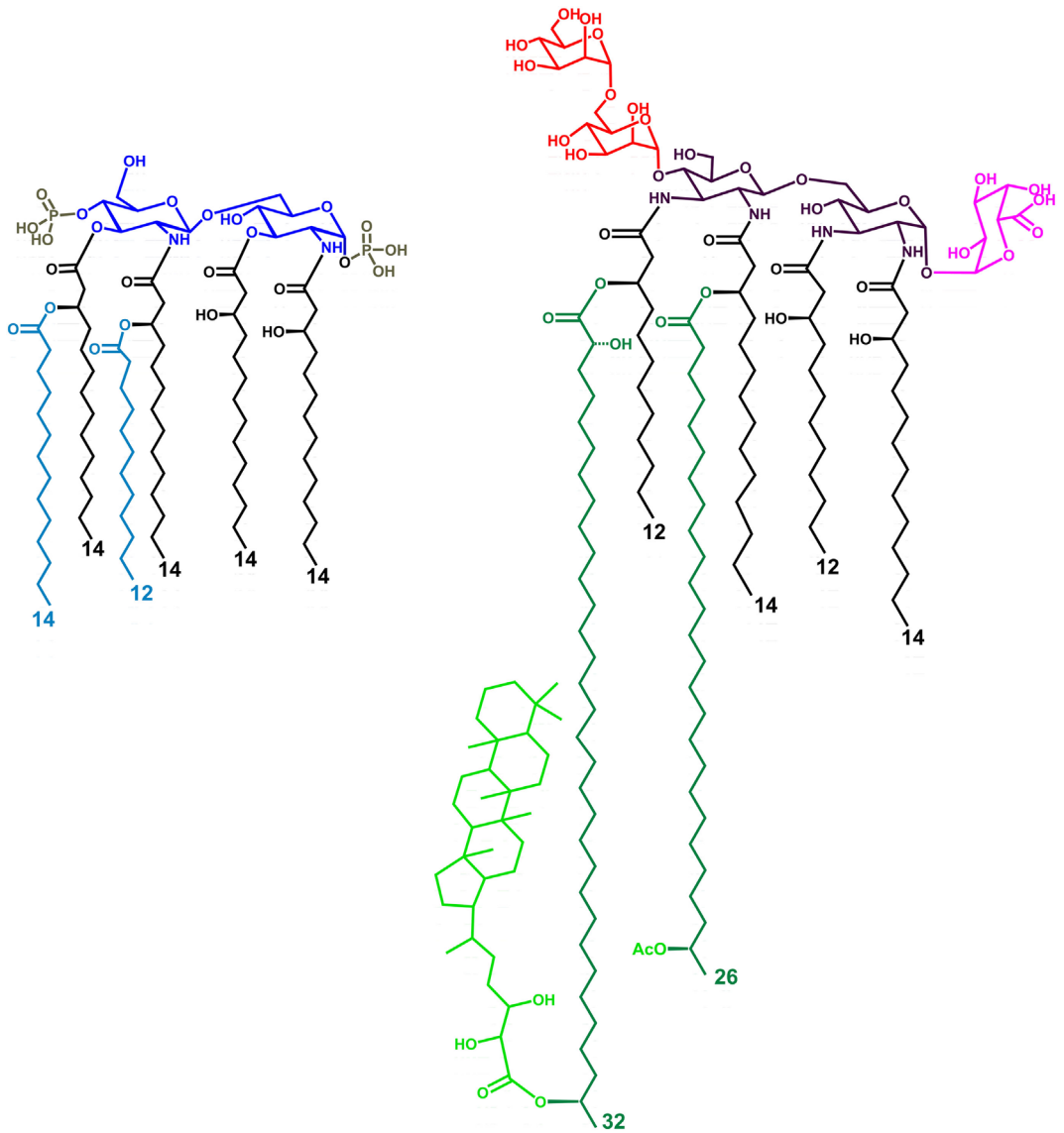


FIGURE 1 | Structure of the LPS lipid A from *Escherichia coli* (left) and *Bradyrhizobium* strains (right, HOLA). The tetra-acylated lipid A from *E. coli* (lipid IV_A) lacks the two secondary acyl chains (light blue colored in the figure). HF-LA refers to hopanoid-free *Bradyrhizobium* lipid A and is devoid of the Hopanoid moiety (light green). The hexa-acylated lipid A from *E. coli* contains a *bis*-phosphorylated glucosamine disaccharide backbone, asymmetrically substituted by six acyl chains. *Bradyrhizobia* lipid A is mainly constituted of a mixture of hexa- and hepta-acylated species, possesses a 2,3-diaminoglucose (DAG) disaccharide backbone, a galacturonic acid residue on the vicinal DAG and an α-(1→6)-mannose disacchaccaride on the distal DAG. The acyl chains are asymmetrically distributed on the sugar skeleton; of the two secondary very long-chain fatty acids present, one is not stoichiometrically substituted at ω-1 position by a hopanepolyol acid.

plasmid, pRLTK (as an internal control). The day after the cells were incubated with different concentrations of *Bradyrhizobium* lipid A or LPS [1, 10, and 100 ng/mL; the LPS and the lipid A preparations were obtained as previously described (21–24)], or with purified *E. coli* LPS (LPS-EB ultrapure; InvivoGen) or with synthetic lipid IV_A used at the same concentrations as above, for 6 h to analyze NF-κB activity (Dual Luciferase Reporter Assay System, Promega) and to measure CXCL-8 release (DuoSet R&D System). For the competition assay, HEK 293-TLR4/MD2-CD14 cells were primed with 1, 10, and 100 ng/mL of *Bradyrhizobium* LPS or lipid A, or lipid IV_A or with *Shigella flexneri* hexa-acylated LPS for 1 h and then exposed to *E. coli* LPS (10 and 100 ng/mL) for 4 h (30). After this time, NF-κB activity and CXCL-8 production were measured.

BMDMs Isolation, Culture, and Stimulation

C57BL/6 mice were purchased from Charles River (Charles River ITALY). BMDMs were derived from the bone marrow cells collected from 5-week-old female mice, as already reported (31). Animal studies were conducted according to protocols approved by the University of Rome La Sapienza and adhered strictly to the Italian Ministry of Health guidelines for the use and care of experimental animals.

BMDMs were differentiated during 7 days in RPMI 1640 (Lonza, Italy), supplemented with 10% of heat-inactivated FBS (HycloneTM, Euroclone, Italy), 1% di L-glutamine (Lonza, Italy), 1% sodium pyruvate (Lonza, Italy), 1% NEAA (Lonza, Italy), 0.5% 2-ME (Gibco, Italy), and 40 ng/mL macrophage colony-stimulating factor (M-CSF; Miltenyi Biotec). BMDMs were seeded into 24-well plate (5×10^5 cells per well) and were incubated with different concentrations of *Bradyrhizobium* LPS or lipid A (1, 10, or 100 ng/mL) or with *E. coli* LPS or with lipid IV_A at the same concentrations as above for 6 h. Where necessary, polyinosinic-polycytidylic acid, Poly (I:C) (Invivogen) was used at the concentration of 5 μg/mL. After this time the supernatants were collected and tumor necrosis factor TNF release was measured through ELISA (DuoSet R&D System). For the competition assay, BMDMs were pre-incubated with *Bradyrhizobium* LPS or lipid A (10, or 100 ng/mL) or with lipid IV_A at the same concentrations as above for 1 h and then exposed to *E. coli* LPS (10 ng/mL) for 4 h. After this time, TNF and CXCL-1 release was quantified *via* ELISA.

RNA Extraction and *q*PCR Analysis of *ifn*-β Expression

Total RNA was extracted from unstimulated or LPS or treated or Poly (I:C)-treated C57BL/6 BMDMs through Trizol reagent (Invitrogen) according to the manufacturer's instructions. RNA was converted to cDNA using High Capacity cDNA Archive kit (Applied Biosystems, Monza, Italy) and random primers, and finally amplified using Power SYBR 17 Green PCR Master Mix (Applied Biosystem,). The $2^{-\Delta\Delta Ct}$ method was applied to analyze the relative changes in expression profiling of interest genes, as already reported (32). Values were normalized to the internal *tbp* gene control. Primers for *q*PCR are for *ifn*-β: F-TCCGAGCAGAGATCTTCAGGAA; R-TGCAACCACCACTCATTCTGAG. For *tbp*: F-CTG GAA TTG TAC CGC AGC TT; R-TCC TGT GCA CAC CAT TTT TC.

Human Peripheral Blood Mononuclear Cells (PBMCs) Isolation and Stimulation

Peripheral blood mononuclear cells were isolated from buffy coats obtained by the blood bank of Sapienza University from healthy adult volunteers (blood donors) following written informed consent. CD14+ monocytes were isolated from PBMCs using the MACS system (Miltenyi Biotec, Bergisch Gladbach, Germany) and cultured in complete RPMI 1640 medium (Lonza, Italy, Milan), supplemented with 10% of heat-inactivated FBS (HycloneTM, Euroclone, Italy, Milan), 1% di L-glutammine (Lonza), 1% of non-essential amino acid solution (NEEA—Lonza), 1% sodium pyruvate (Lonza), penicillin 100 U/mL–streptomycin 100 μg/mL (Lonza), and 0.1% di 2-ME (Gibco, Italy). CD14+ monocytes were seeded at the concentration of 5×10^5 cells/well in 12-multiwell plate and exposed to 0.05, 0.5, 1, and 10 ng/mL of LPS or lipid A from *Bradyrhizobium* or to commercial *E. coli* O111:B4 or to synthetic lipid IV_A at same concentrations as above for 12 h. Cell supernatants were then collected and processed for ELISA to measure the levels of TNF and IL-6.

Cytokine Measurement

Murine and human cytokines were determined in supernatants of stimulated cells by using R&D Systems DuoSet ELISA kits according to the manufacturers' instructions.

Molecular Modeling

Structure Construction

The 3D structures of both the hopanoid-containing and HOLA and HF-LA were built with PyMOL molecular graphics and modeling package based on the saccharide backbone of *E. coli* LPS retrieved from the PDB ID 3FXI. Atoms were modified and added accordingly and bond type and length were carefully selected and revised. The geometry of these two structures was further optimized with Maestro under the OPLS3 force field. The antagonist conformation of the hTLR4/MD-2 complex was assembled by merging the ectodomain of TLR4 from RCSB (www.rcsb.org) PDB ID 3FXI and MD-2 from PDB ID 2E59. The latter was aligned to the spatial coordinates of the MD-2 present in 3FXI and solvent, ligands, and ions were removed.

Structure Optimization

Hydrogen atoms were added to the X-ray structures using the preprocessing tool of the Protein Preparation Wizard of the Maestro package, and then the structures went through a restrained minimization under the OPLS3 force field with a convergence parameter to RMSD for heavy atoms kept default at 0.3 Å.

Docking Procedure

Gasteiger charges were computed and assigned with AutoDockTools 1.5.6 to both the proteins and the ligands. Both HOLA and HF-LA were left flexible by allowing some appropriately selected dihedral angles to rotate whereas the receptor was always kept completely rigid. The docking was performed with AutoDock Vina (33). A cubic docking box of 60 Å in size and 1 Å in spacing was defined. The box was centered equidistant to the geometric center of residues Arg90 (MD-2), Arg96 (MD-2), and Arg264 (TLR4).

Parameterization

The full lipid structures were split into residues to facilitate and homogenize the parameterization process. The partial charges and atom types of the 4-substituted and the 6-substituted 2,3-diamino-2,3-dideoxy-glucose (DAG) monosaccharides composing the oligosaccharide backbone were established respectively based on residues 4YB (4-substituted GlcNAc) and 6YA (6-substituted GlcNAc) of the GLYCAM force field (34). The partial charges and parameters for the two mannose (Man) residues and the galacturonic acid (GalA) were retrieved from the GLYCAM force field respectively under the name 0MA, 6MA, and 1OA. Partial charges for the primary and secondary acylation as well as for the hopanoid residue were derived, with the help of antechamber (31), following the standard GAFF procedure described in the AMBER manual and the parameters were assigned by the GAFF force field.

MD Simulations

All MD simulations were performed with AMBER14 (35), the protein was described by the ff14SB all-atom force field (36), the pentasaccharide backbone of the BTAi1 lipid A by the GLYCAM_06j-1 force field (34) and the other constituents of the lipid A (the lipid chains and the hopanoid moiety) were parameterized with the General Amber Force Field (GAFF) (37). The simulation box was designed such as the edges are distant of at least 10 Å of any atoms. The system was solvated with the TIP3P water molecules model. One Na^+ ion was added to counterbalance the negative charge of the galacturonate group. All the simulations were performed with the same equilibration and production protocol. The equilibration protocol contains height sequential steps. The first one consists of 1,000 steps of steepest descent algorithm followed by 7,000 steps of conjugate gradient algorithm; a 100 kcal $mol^{-1}.Å^{-2}$ harmonic potential constraint is applied on both the proteins and the ligand. In the fourth subsequent steps, the harmonic potential is progressively lowered (respectively to 10, 5, 2.5, and 0 kcal $mol^{-1}.Å^{-2}$) for 600 steps of conjugate gradient algorithm each one. In the sixth step, the system is heated from 0–100 K by a Langevin thermostat in the canonical ensemble (NVT) under a 20 kcal $mol^{-1}.Å^{-2}$ harmonic potential restraint on the proteins and the ligands. The next step heats up the system from 100–300 K in the isothermal-isobaric ensemble (NPT) under the same restraint condition than the previous step. In the last step, the same parameters are used to simulate the system for 100 ps but no harmonic restraint is applied. At this point, the system is ready for the production run, which is performed in the NPT ensemble.

RESULTS

Immunomodulatory Properties of *Bradyrhizobium* LPS and Lipid A

Immunological Tests of *Bradyrhizobium* LPS and Lipid A on HEK293 hTLR4 Cell Line

To evaluate the immunological impact of *Bradyrhizobium* LPS and eventually lipid A, we first analyzed their effect at different concentrations (1, 10, and 100 ng/mL) in the HEK293 cell line (38), stably transfected with human CD14/MD-2/TLR4. Similar concentration of the hexa-acylated, highly stimulatory LPS of *E. coli* O111:B4 and the synthetic lipid IV_A were used as agonistic and antagonistic controls, respectively. Likewise, untreated cells were considered as the negative control in all the experiments shown. Stimulation of HEK293 hTLR4 was carried out for 6 h (**Figures 2A,B**).

With a parallel approach, we used HEK293 hTLR2 cells to assess whether the biological activity of *Bradyrhizobium* LPS could be extended to TLR2, in addition to TLR4 as described for a limited number of LPS (Figure S1A in Supplementary Material) (39). Furthermore, assessment of the *Bradyrhizobium* LPS in this cell model could rule out a potential role for lipopeptides L(Ps) contaminating LPS. In HEK293 hTLR2 cells, Pam3CSK4 (Pam3) (500 ng/mL), a synthetic triacylated lipopeptide (LP) that mimics the acylated amino terminus of bacterial LP, represented the positive control for a TLR2 ligand. Cells treated with *E. coli* LPS, as above, or untreated cells were used as the negative controls. Activation of NF-κB and Chemokine (C-X-C motif) ligand 8 (CXCL-8, also known as IL-8) production was measured after 6 h of stimulation in both assays. As shown in **Figures 2A,B**, stimulation of HEK293 hTLR4 with *Bradyrhizobium* LPS and lipid A stimulation elicited a poor NF-κB activation and CXCL-8 production, which were significantly lower with respect to the values induced by *E. coli* LPS stimulation (for NF-κB and CXCL-8, *Bradyrhizobium* LPS at 10 and 100 ng/mL vs. *E. coli* LPS at same concentrations $p < 0.001$). No activation of NF-κB and CXCL-8 production (Figures S1A,B in Supplementary Material) were observed upon HEK293 hTLR2 stimulation with *Bradyrhizobium* LPS and lipid A, suggesting that neither LPS nor lipid A could trigger the TLR2-mediated signaling.

Immunological Impact of *Bradyrhizobium* LPS and Lipid A on $CD14^+$ Derived Monocytes Derived From PBMCs

The low immunological impact of *Bradyrhizobium* LPS and lipid A was also confirmed in human CD14+ derived monocytes derived from PBMCs, which express all TLRs. Cells were exposed to *Bradyrhizobium* LPS and lipid A at the concentration of 0.05, 0.5, 1, and 10 ng/mL for 12 h. *E. coli* LPS was tested in parallel at same concentrations. The release of the pro-inflammatory cytokines TNF and interleukin-6 (IL-6) was assessed as readout. The results are shown in **Figures 2C,D**. In agreement to what observed in the HEK293 hTLR4 cell model, with *Bradyrhizobium* lipid A the release of these cytokines was poorly detectable at any concentration tested while 10 ng/mL *Bradyrhizobium* LPS clearly induced TNF and IL-6, albeit both were significantly lower than those elicited by *E. coli* LPS at the same concentration (for TNF, *Bradyrhizobium* LPS at 1 and 10 ng/mL vs. *E. coli* at same LPS concentration $p < 0.001$; for IL-6, all *Bradyrhizobium* LPS concentrations vs. *E. coli* all concentrations $p < 0.001$).

Immunological Impact of *Bradyrhizobium* LPS and Lipid A on BMDMs From C57BL/6 Cells

To strengthen the results obtained in the HEK293 cell models and PBMCs, we also used BMDMs from C57BL/6 wild-type mice or from C57BL/6 knockout mice for *Tlr2* (*Tlr2*−/−) or *Tlr4* (*Tlr4*−/−) (Figures S1C,D in Supplementary Material). Murine macrophages were exposed to *Bradyrhizobium* LPS and lipid A at 1, 10, and 100 ng/mL. Pam3 (1 μg/mL) and *E. coli* LPS at the same concentrations as above, were the positive controls for TLR2 and TLR4, respectively. After 6 h stimulation, TNF production was quantified. In wild-type macrophages, stimulation with either *Bradyrhizobium* LPS or lipid A induced significantly less TNF compared to stimulation with Pam3 (all *Bradyrhizobium* LPS concentrations vs. *E. coli* all concentrations $p < 0.001$) as shown in **Figure 2E**. As observed in PBMCs, *Bradyrhizobium* LPS and lipid A induced a scantly amount of this cytokine (all *Bradyrhizobium* LPS and lipid A concentrations vs. *E. coli* all concentrations $p < 0.001$).

When the same experimental set-up was applied to murine macrophages of *tlr2*−/− or *tlr4*−/− mice, we found that the

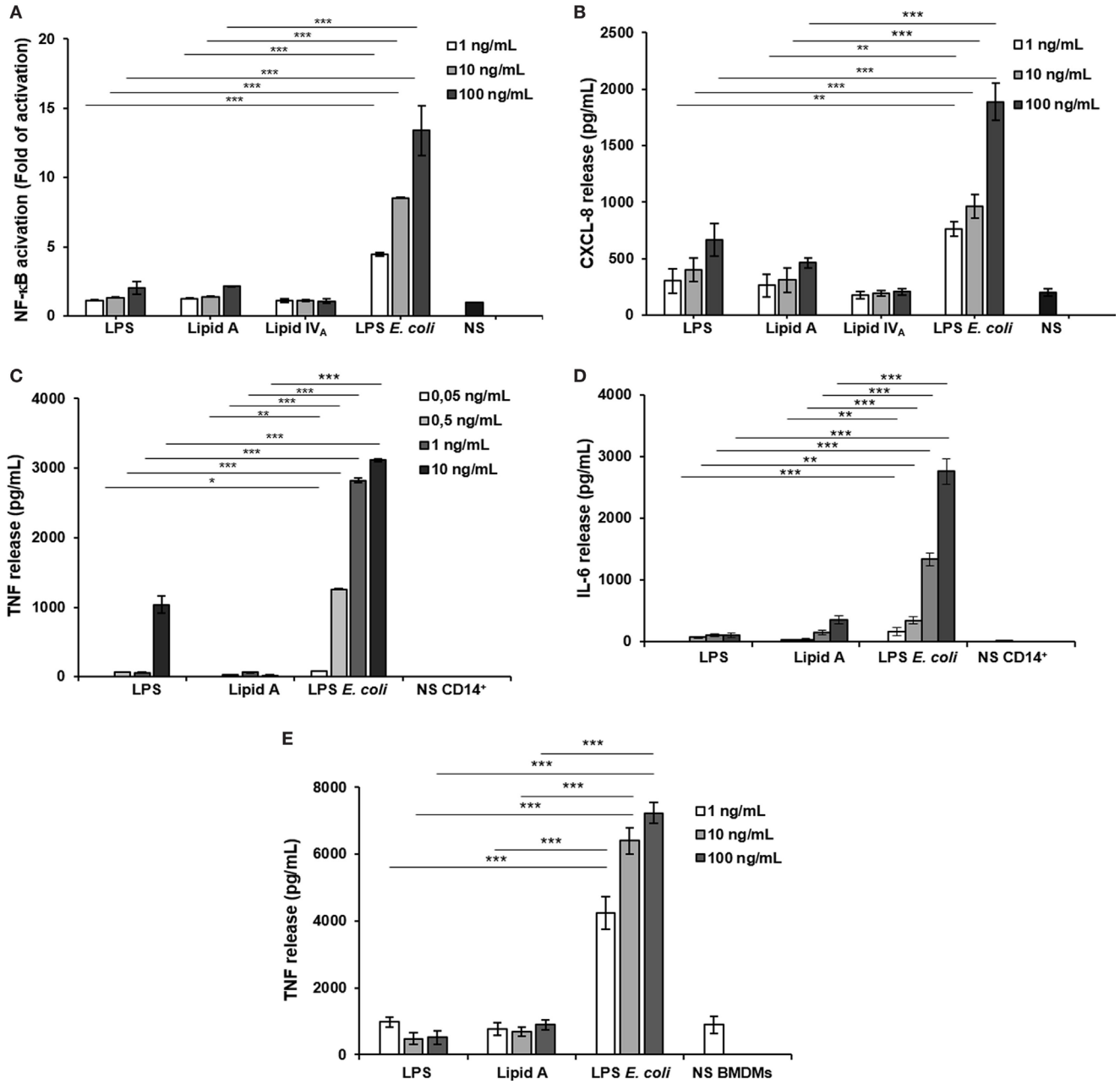


FIGURE 2 | Analysis of immunopotential of *Bradyrhizobium* LPS/lipid A in HEK293 hTLR4 cell line, monocytes, and BMDMs. Activation of nuclear factor kappa B **(A)** and CXCL-8 production **(B)** in HEK 293 hTLR4/MD2-CD14 stimulated with *Bradyrhizobium* LPS/lipid A at the concentration of 1, 10, and 100 mg/mL for 6 h. Commercial hexa-acylated *Escherichia coli* LPS or synthetic lipid IV_A were used at the same concentrations as positive and negative control, respectively. tumor necrosis factor TNF **(C)** and IL-6 **(D)** release in cell-free supernatants of monocytes stimulated with *Bradyrhizobium* LPS/lipid A at the concentration of 0.05, 0.5, 1, and 10 mg/mL for 12 h. Commercial hexa-acylated *E. coli* LPS was used at the same concentrations as control. TNF **(E)** production in BMDMs stimulated with *Bradyrhizobium* LPS/lipid A at the concentration of 1, 10, and 100 mg/mL for 6 h. Abbreviation: NS: not stimulated. Data are expressed as mean ± SD of three independent experiments in triplicate. $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$, after Student's *t*-test.

absence of *tlr2* prevents the production of TNF upon Pam3CSK stimulation but not following LPSs and lipid A stimulation, as expected. On the contrary, in *tlr4*−/− mice TNF production was null and high following LPSs/lipid A and Pam3 stimulation, respectively (Figures S1C,D in Supplementary Material). These results confirm those achieved in the HEK293 cell models: i.e., the reduced immunostimulatory of *Bradyrhizobium* LPS and lipid A is strictly dependent on TLR4 and not on TLR2.

Downstream TLR4-mediated LPS signaling two pathways could be activated. The adaptor protein MyD88 engages a pathway leading to the production of pro-inflammatory cytokines, such as TNF and CXCL-1. The other pathway involves the

proteins Mal (also called TIRAP), TRIF, and TRAM, allowing the expression of genes encoding type 1 interferon and interferon-associated genes (40, 41). Therefore, we faced the question of whether *Bradyrhizobium* LPS and/or lipid A could activate the Trif pathway. With this aim, BMDMs were stimulated as above with 1, 10, or 100 ng/mL of *Bradyrhizobium* lipid A or LPS. *E. coli* LPS and lipid IV_A were used at same concentrations as a control. Polyinosinic-polycytidylic acid, Poly (I:C)—a synthetic analog of double stranded RNA—which acts as TLR3 agonist was used at the concentration of 5 μg/mL to elicit the Trif pathway. After 6 h of stimulation, we measured the release of the cytokine interferon inducible protein 10 (IP-10), which is induced by the axis LPS-TRIF-IRF3-IFN (42) and the chemokine RANTES (CCL-5,) which is expressed through the involvement of IRF3 (43, 44). *Bradyrhizobium* lipid A and lipid IV_A did not elicit any IP-10 release while *Bradyrhizobium* LPS at the concentration of 100 ng/mL could induce a low production of IP-10, which was significantly lower than those observed with *E. coli* LPS at the concentrations of 10 and 100 ng/mL and Poly (I:C) (for both $p < 0.001$), as shown in **Figure 3A**. The same trend was observed for CCL-5 (**Figure 3B**). As IFN-β release can be undetectable upon BMDM stimulation with the fully immunocompetent hexa-acylated *E. coli* LPS (30) we directly proceeded to analyze the mRNA levels for this cytokine. BMDMs were stimulated with 1, 10, or 100 ng/mL of *Bradyrhizobium* LPS or lipid A or with *E. coli* or with lipid IV_A at the same concentrations. Poly (I:C) was used as above. After 3 h of stimulations BMDMs were processed to measure the levels of *ifn*-β RNA through *q*PCR. In accordance with results of IP-10 and CCL-5 release, *ifn*-β RNA was undetectable with *Bradyrhizobium* lipid A while *Bradyrhizobium* LPS at the concentration of 100 ng/mL could trigger a low expression of *ifn*-β (**Figure 3C**). As expected, *E. coli* LPS and Poly (I:C) determined high levels of *ifn*-β RNA. Definitely, these data suggest that *Bradyrhizobium* LPS is a poor elicitor of the Trif pathway.

Competition Tests of *Bradyrhizobium* LPS and Lipid A on HEK293 hTLR4 and on C57BL/6 BMDMs Cell Lines

Finally, as some under-acylated lipid A (45) and, more recently, unusual LPS-containing long FA chains (46), have been reported to show an inhibitory activity against endotoxically active LPS (47), we assessed the ability of *Bradyrhizobium* LPS to affect the TLR4-mediated signaling triggered by the hexa-acylated, fully immunocompetent *E. coli* LPS. With this aim, HEK 293

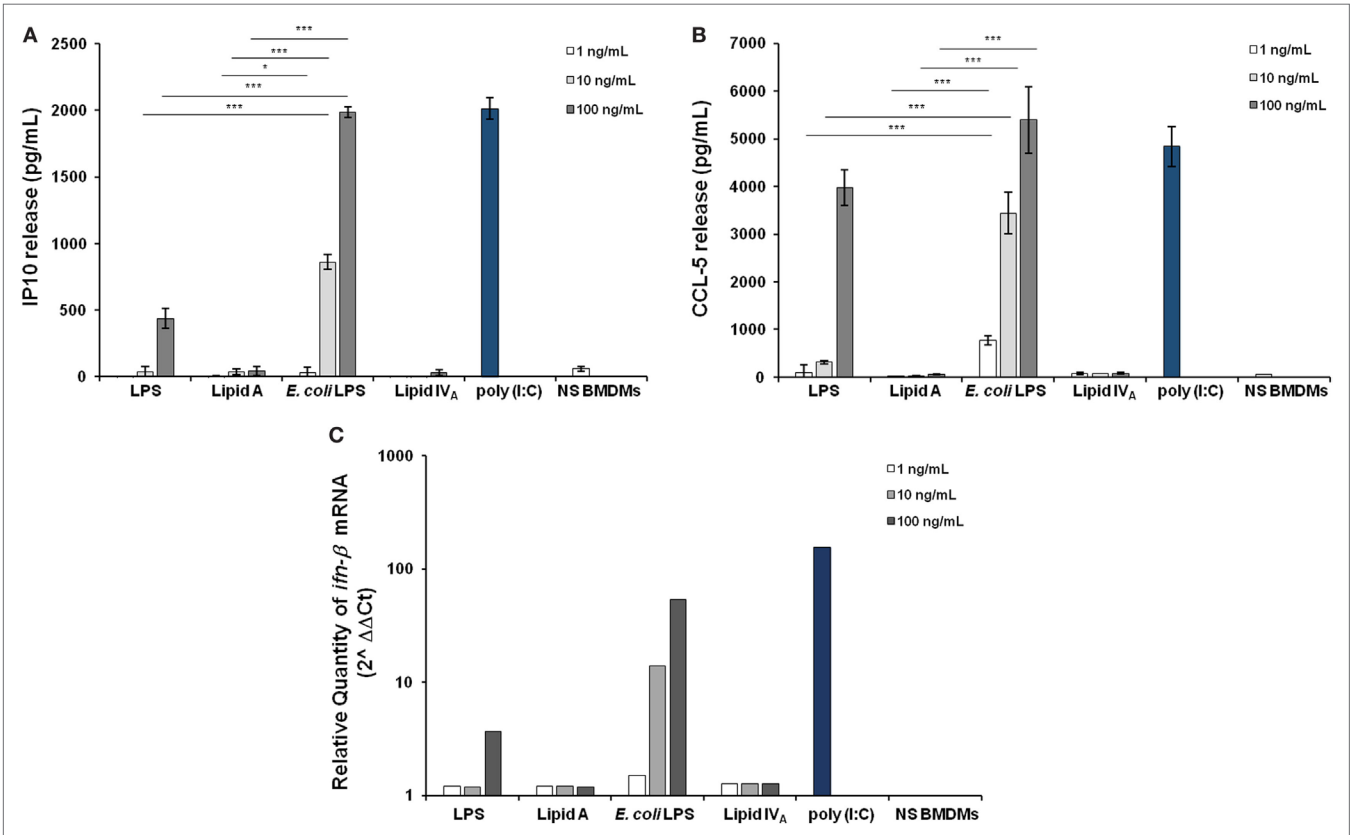


FIGURE 3 | Assessment of the involvement of Trif pathway upon stimulation of BMDMs with *Bradyrhizobium* LPS/lipid A. Inducible protein 10 **(A)** and CCL-5 production **(B)** in BMDMs stimulated with *Bradyrhizobium* LPS/lipid A at the concentration of 1, 10, and 100 ng/mL for 6 h. Commercial hexa-acylated *Escherichia coli* LPS or synthetic lipid IV_A were used at the same concentrations. The TLR3 agonist poly (I:C) (5 μg/mL) was used as a positive control of Trif pathway activation. Data are expressed as mean ± SD of three independent experiments in triplicate. $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$, after Student's *t*-test. **(C)** *q*PCR of *ifn*-β mRNA following 3 h of stimulation of BMDMs with LPS/lipid A at the same concentrations, as above. Results are normalized to the internal TBP gene control and are presented on a logarithmic scale as the ratio of gene expression between stimulated and unstimulated BMDMs.

hTLR4 cells were pre-incubated for 1 h with *Bradyrhizobium* LPS and stimulated with 10 and 100 ng/mL of *E. coli* LPS for 4 h (**Figures 4A–D**). NF-κB activation and CXCL-8 production were quantified after this time. The synthetic tetra-acylated lipid IV_A was used in parallel under the same conditions as for *Bradyrhizobium* LPS as a control of the inhibitory effect. A hexa-acylated LPS of *Shigella flexneri* (30) was used in parallel. Untreated cells and cells stimulated with the *E. coli* LPS at 10 and

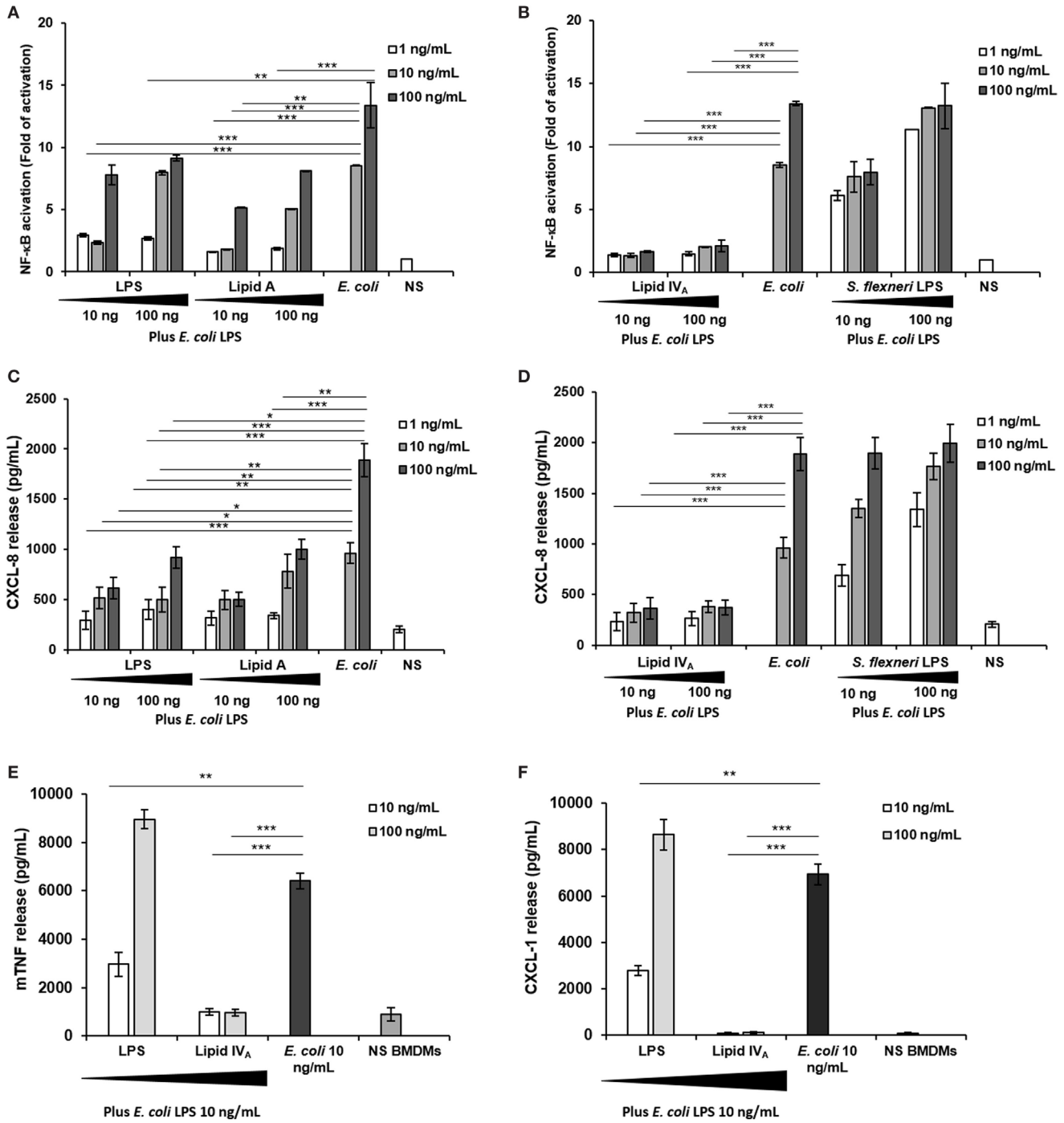


FIGURE 4 | Competition assay: **(A–D)** HEK293 hTLR4 cell line; **(E,F)** BMDMs. **(A,B)** Fold of nuclear factor kappa B activation and **(C,D)** CXCL-8 release following priming with 10 and 100 ng/mL of *Bradyrhizobium* LPS or lipid A, or with lipid IV_A or with *Shigella flexneri* hexa-acylated LPS for 1 h and then exposed to *Escherichia coli* LPS (10 and 100 ng/mL) for 4 h. Stimulation with the only *E. coli* LPS (10 and 100 ng/mL) for 5 h was used as a control. **(E,F)** BMDMs were stimulated with 10 and 100 ng/mL of *Bradyrhizobium* LPS or with lipid IV_A during 1 h and then incubated with 10 ng/mL of *E. coli* LPS for 4 h. After this time TNF and CXCL-1 release were quantified. Stimulation with the only *E. coli* LPS (10 ng/mL) for 5 h was used as a control. Data are expressed as mean ± SD of three independent experiments in triplicate. $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$, after Student's *t*-test.

100 ng/mL for 4 h were the control in this experiment. Under these experimental conditions, *Bradyrhizobium* LPS showed an inhibitory activity on *E. coli* LPS at all the concentrations tested (NF-κB values of *Bradyrhizobium* LPS + *E. coli* LPS vs. *E. coli* LPS alone, for NF-κB; *Bradyrhizobium* LPS at 1 and 10 ng/mL $p < 0.001$; *Bradyrhizobium* LPS at of 100 ng/mL $p < 0.01$).

Finally, we evaluated whether *Bradyrhizobium* LPS could also interfere with TLR4 signaling in murine cells which have been reported to be less sensitive to the degree of lipid A acylation (48, 49). Therefore, BMDMs were stimulated with 10 and 100 ng/mL of *Bradyrhizobium* LPS for 1 h and then exposed to 10 ng/mL of *E. coli* LPS for 4 h (**Figures 4E,F**). Unstimulated cells and BMDMs stimulated with 10 ng/mL *E. coli* LPS for 5 h were the controls. The production of TNF and CXCL-1 was measured. As shown in **Figures 4E,F** under these conditions only the concentration of 10 ng/mL exerted an inhibitory effect on the *E. coli* LPS ($p < 0.01$).

Molecular Modeling of *Bradyrhizobium* Lipid A Binding to MD-2/TLR4

We performed computational studies to identify the possible binding modes and understand the dynamic behavior of *Bradyrhizobium* lipid A (**Figure 1**) in complex with human MD-2/TLR4. Since the experimental samples contain different derivatives, we studied both hopanoid-containing and hopanoid-free *Bradyrhizobium* lipid A (HOLA and HF-LA respectively, **Figure 1**). Docking results were evaluated based on the predicted binding score and on the apparent degree of similarity with *E. coli* LPS and lipid IV_A as known from their X-ray crystallographic structures accessible under the PDB accession codes 3FXI and 2E59, respectively. We considered both the insertion of the FA chains into the MD-2 pocket and the positioning of the disaccharide backbone. In addition, we systematically discarded the poses in which at least one of the saccharide-bearing acyl chains was rotated such as that the amide groups connecting the saccharide to the lipid chains were facing the opposite direction of the binding pocket. This orientation causes a large portion of the lipid chains to be exposed to the solvent, which we consider unlikely.

Docking Calculations of HF-LA and HOLA in MD-2

We started by carrying out docking calculations of HF-LA and HOLA in MD-2. Plausible binding modes were obtained with most of the FA chains inserted inside the MD-2 pocket, while the sugar moieties interact at its rim. As for the interactions, in the case of HF-LA, the two VLCFA are often fully accommodated inside the MD-2 cavity where they are surrounded by hydrophobic residues, such as Val24, Ile32, 46, 63, 94, 117, 153, and Leu61, 78, leaving space for only two shorter lipid chains to enter the pocket. One of the two remaining shorter chains is often directed toward Phe126. The other one is placed in a small corridor pointing at Ser103 (**Figure 5**).

In the case of HOLA, the hopanoid moiety lies at the rim of the MD-2 pocket close to the residues Asp101, Tyr102, and Ser118 (**Figure 5**) or at a completely distinct location close to residue Lys125 and Phe126. In many docked poses, the hopanoid moiety remains on a surface outside or at the rim of the MD-2 pocket. Some low-score docking poses feature the hopanoid moiety in the hydrophobic pocket, proving that it could sterically be accommodated inside MD-2. However, in these poses, the pentasaccharide backbone is accommodated further away from the binding pocket, on a loop followed by a β-sheet formed by residues 87–91 (Figure S2 in Supplementary Material). This is likely due to the steric constraints inherent to the hopanoid moiety being inserted in the pocket. In addition, it occupies a consequent volume and seems to obstruct the passage for the lipid chains, resulting in poses in which at least three acyl chains are left outside the pocket. These results suggest that the hopanoid moiety may not play a particularly important role in the effective binding of HOLA to MD-2, and thus its presence might not be necessary for *Bradyrhizobium* to exert its antagonist activity.

Docking Calculations and MD Simulation of *Bradyrhizobium* Lipid A Into MD-2/TLR4 Model

In a second approach, docking calculations of HOLA were performed into the MD-2/TLR4 model in the antagonist conformation reported by us somewhere else (50). Interestingly, when compared with the structure of the MD-2/TLR4 complex in the

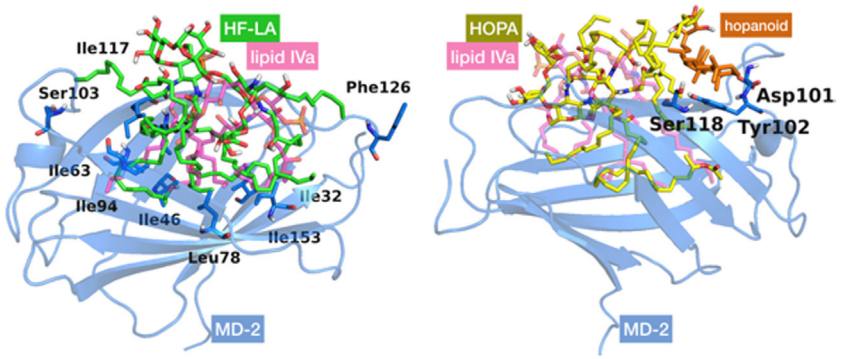


FIGURE 5 | Best docked poses for HF-LA, represented in green sticks (on the left), and HOLA, in yellow sticks and the hopanoid residue in orange (on the right), inside myeloid differentiation protein-2 (MD-2) structure. Lipid IV_A, added for comparison purposes, is depicted in pink CPK colored semi-transparent sticks. MD-2 is in blue semi-transparent cartoon. Some residues mentioned in the text are in sticks with their corresponding individual labeling.

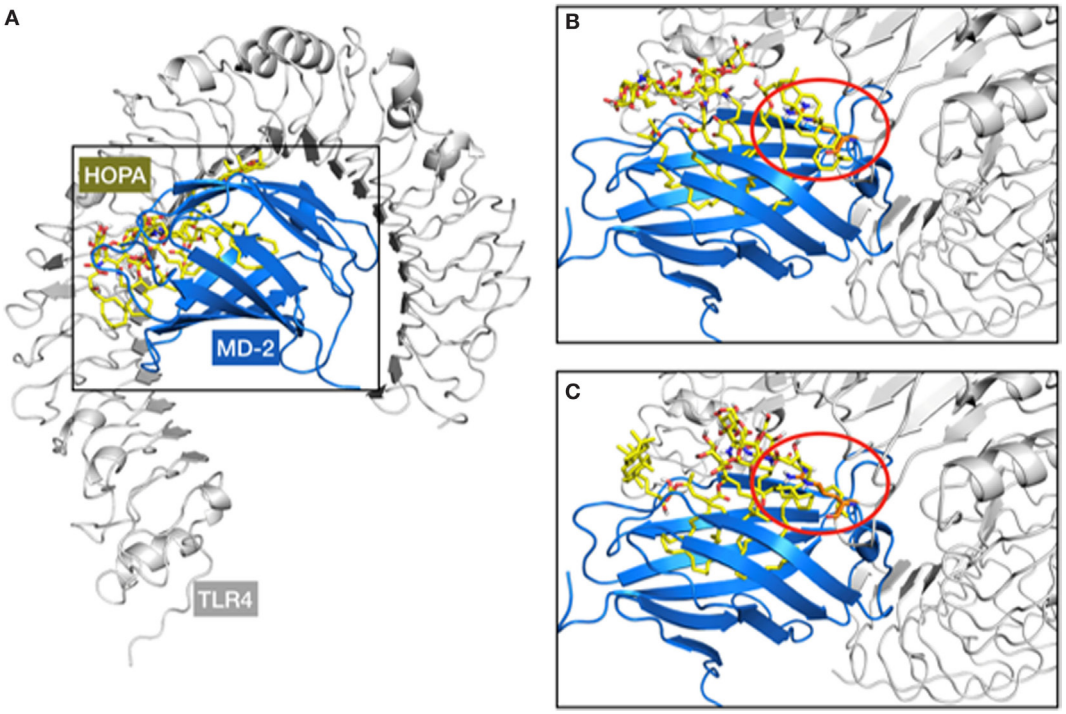


FIGURE 6 | Representation of the steric clash observed over the superimposition of the myeloid differentiation protein-2 (MD-2)/HOLA complex (from docking calculations) to the MD-2/toll-like receptor 4 (TLR4) structure (from PDB ID 3FXI). A general view of the MD-2/TLR4/HOPA complex **(A)** and two examples of steric clashes are given **(B,C)**. TLR4, MD-2, and HOPA are respectively represented in gray cartoon, blue cartoon, and yellow sticks. The TLR4 protruding loop mentioned in the text is marked within the red circle, involving the hopanoid moiety **(B)** and one of the short acyl chains of HOPA **(C)**.

agonist conformation (PDB ID 3FXI), among the non-bonded interactions between the two proteins, a loop of TLR4, composed of amino acids 263–266, protrudes into a MD-2 channel (Figure S2 in Supplementary Material), located approximately between Asp161 and Tyr118. This protrusion is further amplified by the side chain of Arg264 that goes as far as to hover over the MD-2 hydrophobic pocket. This impingement of TLR4 over MD-2 diminishes the space available for ligand interactions in the MD-2/TLR4 complex compared with MD-2 alone. We superimposed TLR4, based on the X-ray crystallographic structure (PDB ID 3FXI), to the docking results from the MD-2-only study and noted a steric incompatibility between the TLR4 protruding loop, and either a lipid chain or the hopanoid moiety from the docked ligand (**Figure 6**). This observation could point to the fact that HOLA and HF-LA carry on their antagonist activity by preventing or impairing the formation of a proper MD-2/TLR4 dimer essential for TLR4 activation.

The hydrophobic interactions taking place inside the hydrophobic pocket were essentially the same as the ones described in the case of MD-2 alone. However, the presence of TLR4, reducing the space available for ligand binding, resulted in very few poses respecting the sugar orientation criterion mentioned above. Two poses of HOLA, in good agreement with lipid IV_A, were selected for MD simulations to further investigate interactions with the receptor and the overall stability of the MD-2/TLR4/ligand complex (**Figure 7**). These two poses are rotated 180° one to the other: in the first one, the HOLA lipid A is oriented as lipid IV_A (PDB ID 2E59) and, in the second one, it is oriented as *E. coli* LPS (PDB ID 3FXI, Figure S2 in Supplementary Material). Along the simulation time, the TLR4 presents important deviation compared to the crystal structure in relation with MD-2, as shown in the RMSD plot (Figure S4 in Supplementary Material). TLR4 displays a tendency to break apart from MD-2 indicating that the presence of the ligand destabilizes the TLR4/MD-2 complex (**Figure 8**).

This observation backs-up the hypothesis that *Bradyrhizobium* lipid A act as antagonists by either preventing complex formation (cf. protruding loop mentioned in the docking study above) or by disturbing the complex stability. In addition, Phe126 remains in its open conformation all along the simulation (Figure S4 in Supplementary Material), this stability was previously associated with antagonist ligands (51).

DISCUSSION

LPS is among the most potent pro-inflammatory compounds known, with its lipid A moiety eliciting the production of host-derived inflammatory mediators. Pyrogenicity and lethality of lipid A strictly relies on a set of structural features which includes the number and distribution of appropriately long acyl chains with respect to the glucosamine disaccharide backbone as well as the occurrence of phosphate units decorating the sugar backbone (52, 53). The possibility to prevent the detrimental effects of toxic LPS by competing lipid A derivatives with a weak or no

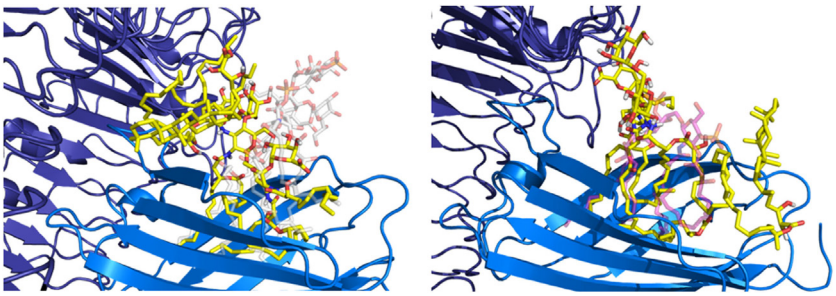


FIGURE 7 | Representation of the two poses of HOPA (yellow) docked into myeloid differentiation protein-2/toll-like receptor 4 (blue/violet), *Escherichia coli* lipid A-like (on the left) and lipid IV_A-like (on the right), selected for MD simulations.

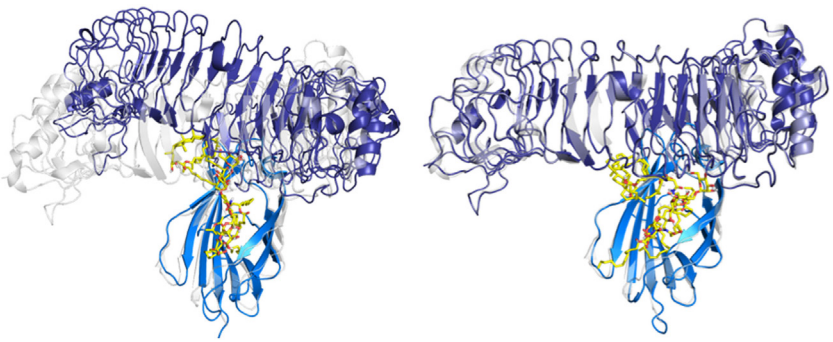


FIGURE 8 | Evolution of the myeloid differentiation protein-2 (MD-2)/toll-like receptor 4 (TLR4)/HOPA complex (blue/violet/yellow) over the MD simulation. MD-2/TLR4 structure from PDB ID 3FXI, represented in semi-transparent cartoon (gray), was superimposed for comparison purposes. On the left: system at $t = 0$ ns, from the docking calculation. On the right: system at $t = 100$ ns of MD simulation.

immunostimulant power is a hot topic with an increasing appeal in several research fields (54, 55). In this context, one of the most studied is the set of synthetic analogs of lipid A of *Rhodobacter capsulatus* or *Rhodobacter sphaeroides* which are known to act as antagonists toward toxic effects of enterobacterial LPS on human cells (56–58).

Within this frame, we have recently shown that *Bradyrhizobium* lipid A present peculiar structural features comprising a pentasaccharide backbone formed by a skeleton of β-(1→6) linked 2,3-diamino-2,3-dideoxy-glucose (DAG) substituted by an α-GalA on the vicinal DAG and by an α-mannose disaccharide linked to the distal β-DAG unit (21–25); the LPS exhibits a heterogeneous blend of lipid A species, in terms of number and nature of acyl chains asymmetrically distributed on the sugar skeleton and contains VLCFA. Furthermore, as a unique peculiarity, *Bradyrhizobium* strains lipid A displays the covalent attachment of a hopanoid molecule to a VLCFA (21).

Given the not human pathogen/human associated nature of *Bradyrhizobium* strains, the unusual structural features of its lipid A, in addition to the still unresolved mechanisms at the basis of rhizobial LPS immunoactivity, prompted the investigation of the immunological properties of such a complex and novel molecule which turned out to act as a very weak agonist of murine and human immune cells. More importantly, we proved that *Bradyrhizobium* LPS is able to potently inhibit the toxic effects of *E. coli* LPS on both murine and human immune systems as a significant decrease in the cytokine release and NF-kB activation were clearly observed when cells were stimulated with both *Bradyrhizobium* LPS and lipid A and then re-stimulated with the toxic *E. coli* or *S. flexneri* LPS. We found that the inhibitory effect is especially evident at low *Bradyrhizobium* LPS concentrations. This is not surprising since this LPS is constituted by several lipid A species as mentioned above. We can hypothesize that this blend could contain one/some species provided of an inhibitory activity and others deprived of this property. By increasing the LPS concentration, the effect of these latter species prevails on that of the antagonistic species thus lowering the inhibitory capacity of this LPS. We observed the same effect with other LPS blends such as that of the intracellular *S. flexneri* (30) on *E. coli* LPS. Lipid A structures such as those of *Pseudomonas aeruginosa*, *Bordetella pertussis*, *Leptospira interrogans*, and *Neisseria meningiditis* are differently recognized by mouse and human TLR4 (48, 49). The acylation degree, the phosphate presence/absence or substitution and other still unknown molecular features of lipid A underline the differential recognition by the human and murine TLR4 complex. In line with this issue, here the inhibitory activity of *Bradyrhizobium* LPS seems to be more evident when tested on human TLR4 than on murine TLR4 (see **Figures 2A,E**) in this

way stressing this difference. Furthermore, we also showed that *Bradyrhizobium* lipid A acts exclusively through TLR4 and do not activate TLR2 at all, in accordance with data from other lipid A strains (59). These immunological properties, as stated above, can be correlated to the peculiar structure of rhizobial LPS which showed uncommon sugar backbone, lacking phosphate decoration and presenting, among the others, VLCFA with a chain length ranging from 26 to 32 carbon atoms. In support to the hypothesis that VLCFA are involved in the potent TLR4 inhibitory activity observed for some rhizobial LPSs, a recent study showed that the human pathogen *Bartonella quintana*, expressing a penta-acylated lipid A decorated by one VLCFA [namely 26:0 (25-OH)], was able to potently block TLR4 activation by rapidly and protractedly binding the receptor complex (46). Interestingly, other intracellular pathogens belonging to *Brucella* (60, 61) and *Legionella* (62, 63) species and phylogenetically related opportunistic bacteria like *Ochrobactrum* contain VLCFA in their lipid A; in all these species, the low immunopotential of their lipid A LPS likely favors the escape from the innate immune system and the intracellular entry, as already demonstrated for other intracellular living bacteria as *Shigella* (30).

To shed light on this behavior, we investigated the molecular basis of the *Bradyrhizobium* lipid A binding to the MD-2/TLR4 complex (64). Our computational data demonstrated that the occurrence of VLCFA, likely fully accommodated inside the MD-2 cavity, may be responsible for the antagonistic properties of *Bradyrhizobium* lipid A by impairing the proper complexation of the TLR4/MD-2 dimer or potentially by destabilizing the complex itself, and, furthermore, do not point toward a primary role of the hopanoid moiety in the biological activity regarding TLR4 signaling. Our results suggest that the TLR4 signaling modulation is likely to occur by direct interaction with the TLR4/MD-2 complex, both in its hopanoid-containing and hopanoid-free forms. We indeed derived plausible binding modes of both HOLA and HF-LA to the MD-2/TLR4 system, demonstrating that *Bradyrhizobium* lipid A can act as antagonist by either preventing complex formation or by disturbing the complex stability, this accounting for the potent activity antagonizing *E. coli* LPS binding to the MD-2/TLR4 complex thus inhibiting its toxic effects. A clear understanding, at atomic level, of the molecular mechanisms leading to TLR4 activation/inhibition upon binding of *Bradyrhizobium* LPS can be of help in future prediction of the immunomodulatory properties of LPSs, and consequently of their potential use in biomedical applications, based on the chemical features of the lipid domain.

In summary, our work certainly confirmed that the rhizobia LPS world is a promising source of MD-2/TLR4 immunomodulators, that can be instrumental for the rational development of endotoxin-based therapeutics and/or vaccine adjuvants. Indeed, weak agonists, as *Bradyrhizobium* LPS has shown to be, are typically desired to be used as adjuvants in vaccine production, whereas antagonists, as stated above, are being sought as inhibitors of TLR4-dependent signaling to fight against sepsis (65). This becomes even more appealing under another point of view focused on the growing body of information describing the involvement of LPS and TLR4 in development of strongly emerging pathologies such as allergies. Indeed, it has been demonstrated that exposure to high concentrations of LPS induces allergic airway inflammation symptoms and disease *via* TLR4 signaling (66, 67). On the contrary, it has been demonstrated that environmental exposure to LPS can exert a protective action against the development of atopy and asthma (10). More interestingly, allergy-protective activity by the hepta-acylated LPS from the farm environmental bacterium *Acinetobacter lwoffii* F78 has been previously described and compared to other structurally different LPSs, such as the canonical hexa-acylated LPS from *E. coli*, which showed apparently no protective properties in concentrations comparable to a farming habitat (68). It would be tempting to say that the identification of new immunomodulatory compounds with a weak immunostimulant activity on TLR4 as well as an inhibitory action of harmful LPSs might also open to prevention of such diseases caused by abnormally exacerbated reactions to environmental factors. Therefore, determining and understanding how structural features of LPSs may affect the activation/modulation of the immune response may provide the mechanism for the fine tuning of the response itself as well as new insights to immunomodulatory processes.

AUTHOR CONTRIBUTIONS

AS conceived the project. AS, M-LB, and SM-S analyzed literature and designed detailed research. All the authors performed their respective experiments. FL, LL-F, J-MB, AS, M-LB, and SM-S wrote the manuscript. All authors analyzed data and revised the manuscript.

FUNDING

The authors acknowledge the European Commission (H2020-MSCA- ETN-642157 TOLLerant project) and Spanish MINECO (CTQ2014-57141-R and CTQ2017-88353-R) for financial support.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.01888/full#supplementary-material.

REFERENCES

1. Raetz CR, Whitfield C. Lipopolysaccharide endotoxins. *Annu Rev Biochem* (2002) 71:635–700. doi:10.1146/annurev.biochem.71.110601.135414
2. Molinaro A, Holst O, Di Lorenzo F, Callaghan M, Nurisso A, D'Errico G, et al. Chemistry of lipid A: at the heart of innate immunity. *Chemistry* (2015) 21:500–19. doi:10.1002/chem.201403923
3. Netea MG, van Deuren M, Kullberg BJ, Cavaillon JM, van der Meer JW. Does the shape of lipid A determine the interaction of LPS with toll-like receptors? *Trends Immunol* (2002) 23:135–9. doi:10.1016/S1471-4906(01)02169-X
4. Di Lorenzo F, Kubik Ł, Oblak A, Lorè NI, Cigana C, Lanzetta R, et al. Activation of human TLR4/MD-2 by hypoacylated lipopolysaccharide from a clinical isolate of *Burkholderia cenocepacia*. *J Biol Chem* (2015) 290(35):21305–19. doi:10.1074/jbc.M115.649087

5. Poltorak A, Ricciardi-Castagnoli P, Citterio S, Beutler B. Physical contact between lipopolysaccharide and toll-like receptor 4 revealed by genetic complementation. *Proc Natl Acad Sci U S A* (2000) 97:2163–7. doi:10.1073/pnas.040565397
6. Ohto U, Fukase K, Miyake K, Satow Y. Crystal structures of human MD-2 and its complex with antiendotoxic lipid IVA. *Science* (2007) 316:1632–4. doi:10.1126/science.1139111
7. Park BS, Song DH, Kim HM, Choi BS, Lee H, Lee JO. The structural basis of lipopolysaccharide recognition by the TLR4-MD-2 complex. *Nature* (2009) 458:1191–5. doi:10.1038/nature07830
8. Meng J, Drolet JR, Monks BG, Golenbock DT. MD-2 residues tyrosine 42, arginine 69, aspartic acid 122, and leucine 125 provide species specificity for lipid IVA. *J Biol Chem* (2010) 285:27935–43. doi:10.1074/jbc.M110.134668
9. Shirey KA, Lai W, Scott AJ, Lipsky M, Mistry P, Pletneva LM. The TLR4 antagonist Eritoran protects mice from lethal influenza infection. *Nature* (2013) 497:498–502. doi:10.1038/nature12118
10. Racila DM, Kline JN. Perspectives in asthma: molecular use of microbial products in asthma prevention and treatment. *J Allergy Clin Immunol* (2005) 116(6):1202–5. doi:10.1016/j.jaci.2005.08.050
11. Noel KD, Duelli DM. Rhizobium lipopolysaccharide and its role in symbiosis. In: Triplett EW, editor. *Prokaryotic Nitrogen Fixation: A Model System of Analysis of a Biological Process*. Wymondham, UK: Horizon Scientific Press (2000). p. 415–31.
12. Vandenplas ML, Carlson RW, Jeyaretnam BS, McNeill B, Barton MH, Norton N, et al. Rhizobium Sin-1 lipopolysaccharide (LPS) prevents enteric LPS-induced cytokine production. *J Biol Chem* (2002) 277(44):41811–6. doi:10.1074/jbc.M205252200
13. Urbanik-Sypniewska T, Choma A, Kutkowska J, Kaminska T, Kandefer-Szerszen M, Russa R, et al. Cytokine inducing activities of rhizobial and mesorhizobial lipopolysaccharides of different lethal toxicity. *Immunobiology* (2000) 202:408–20. doi:10.1016/S0171-2985(00)80043-1
14. Komaniecka I, Zdzisinska B, Kandefer-Szerszen M, Choma A. Low endotoxic activity of lipopolysaccharides isolated from *Bradyrhizobium*, *Mesorhizobium*, and *Azospirillum* strains. *Microbiol Immunol* (2010) 54:717–25. doi:10.1111/j.1348-0421.2010.00269.x
15. Tsukushi Y, Kodo N, Saeki K, Sugiyama T, Koide N, Mori I, et al. Characteristic biological activities of lipopolysaccharides from *Sinorhizobium* and *Mesorhizobium*. *J Endotoxin Res* (2004) 10:25–31. doi:10.1177/09680519040100010301
16. Oldroyd GE, Murray JD, Poole PS, Downie JA. The rules of engagement in the legume-rhizobial symbiosis. *Annu Rev Genet* (2011) 45:119–44. doi:10.1146/annurev-genet-110410-132549
17. Masson-Boivin C, Giraud E, Perret X, Batut J. Establishing nitrogen-fixing symbiosis with legumes: how many rhizobium recipes? *Trends Microbiol* (2009) 17:458–66. doi:10.1016/j.tim.2009.07.004
18. Andam CP, Parker MA. Novel alphaproteobacterial root nodule symbiont associated with *Lupinus texensis*. *Appl Environ Microbiol* (2007) 73(17):5687–91. doi:10.1128/AEM.01413-07
19. Giraud E, Moulin L, Vallenet D, Barbe V, Cytryn E, Avarre JC, et al. Legumes symbioses: absence of Nod genes in photosynthetic bradyrhizobia. *Science* (2007) 316(5829):1307–12. doi:10.1126/science.1139548
20. Zhang YF, Wang ET, Tian CF, Wang FQ, Han LL, Chen WF, et al. *Bradyrhizobium elkanii*, *Bradyrhizobium yuanmingense* and *Bradyrhizobium japonicum* are the main rhizobia associated with *Vigna unguiculata* and *Vigna radiata* in the subtropical region of China. *FEMS Microbiol Lett* (2008) 285:146–54. doi:10.1111/j.1574-6968.2008.01169.x
21. Silipo A, Vitiello G, Gully D, Sturiale L, Chaintreuil C, Fardoux J, et al. Covalently linked hopanoid-lipid A improves outer-membrane resistance of a *Bradyrhizobium* symbiont of legumes. *Nat Commun* (2014) 5:5106. doi:10.1038/ncomms6106
22. Komaniecka I, Choma A, Mazur A, Duda KA, Lindner B, Schwudke D, et al. Occurrence of an unusual hopanoid-containing lipid A among lipopolysaccharides from *Bradyrhizobium* species. *J Biol Chem* (2014) 289(51):35644–55. doi:10.1074/jbc.M114.614529
23. Belin BJ, Busset N, Giraud E, Molinaro A, Silipo A, Newman DK. Hopanoid lipids: from membranes to plant-bacteria interactions. *Nat Rev Microbiol* (2018) 16(5):304–15. doi:10.1038/nrmicro.2017.173
24. Kulkarni G, Busset N, Molinaro A, Gargani D, Chaintreuil C, Silipo A, et al. Specific hopanoid classes differentially affect free-living and symbiotic states of *Bradyrhizobium* diazoefficiens. *MBio* (2015) 6(5):e1251–1215. doi:10.1128/mBio.01251-15
25. Di Lorenzo A, Palmigiano A, Duda KA, Pallach M, Busset N, Sturiale L, et al. Structure of the lipopolysaccharide from the *Bradyrhizobium* sp. ORS285 rfaL mutant strain. *ChemistryOpen* (2017) 6:541–53. doi:10.1002/open.201700074
26. Kannenberg EL, Carlson RW. Lipid A and O-chain modifications cause *Rhizobium* lipopolysaccharides to become hydrophobic during bacteroid development. *Mol Microbiol* (2001) 39:379–91. doi:10.1046/j.1365-2958.2001.02225.x
27. Ferguson GP, Datta A, Baumgartner J, Roop RM, Carlson RW, Walker GC. Similarity to peroxisomal-membrane protein family reveals that *Sinorhizobium* and BrucellaBacA affect lipid-A fatty acids. *Proc Natl Acad Sci U S A* (2004) 101:5012–501. doi:10.1073/pnas.0307137101
28. Brown DB, Huang YC, Kannenberg EL, Sherrier DJ, Carlson RW. An acpXL mutant of *Rhizobium leguminosarum* bv. phaseoli lacks 27-hydroxyoctacosanoic acid in its lipid A and is developmentally delayed during symbiotic infection of the determinate nodulating host plant *Phaseolus vulgaris*. *J Bacteriol* (2011) 193:4766–78. doi:10.1128/JB.00392-11
29. Busset N, Di Lorenzo F, Palmigiano A, Sturiale L, Gressent F, Fardoux J, et al. The very long chain fatty acid (C26:25OH) linked to the lipid A is important for the fitness of the photosynthetic *Bradyrhizobium* strain ORS278 and the establishment of a successful symbiosis with Aeschynomene legumes. *Front Microbiol* (2017) 8:1821. doi:10.3389/fmicb.2017.01821
30. Paciello I, Silipo A, Lembo-Fazio L, Curcuru L, Zumsteg A, Noel G, et al. Intracellular *Shigella* remodels its LPS to dampen the innate immune recognition and evade inflammasome activation. *Proc Natl Acad Sci U S A* (2013) 110:E4345–54. doi:10.1073/pnas.1303641110
31. Wang J, Wang W, Kollman PA, Case DA. Automatic atom type and bond type perception in molecular mechanical calculations. *J Mol Graph Model* (2006) 25:247260. doi:10.1016/j.jmgm.2005.12.005
32. Lembo-Fazio L, Nigro G, Noël G, Rossi G, Chiara F, Tsilingiri K, et al. Gadd45α activity is the principal effector of *Shigella* mitochondria-dependent epithelial cell death in vitro and ex vivo. *Cell Death Dis* (2011) 2:e122. doi:10.1038/cddis.2011.4
33. Trott O, Olson AJ. AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization and multithreading. *J Comput Chem* (2010) 31:455–61. doi:10.1002/jcc.21334
34. Kirschner KN, Yongye AB, Tschampel SM, Daniels CR, Foley BL, Woods RJ. GLYCAM06: a generalizable biomolecular force field. Carbohydrates. *J Comput Chem* (2008) 29:622–55. doi:10.1002/jcc.20820
35. Case David A, et al. Amber 14. (2014).
36. Maier JA, Martinez C, Kasavajhala K, Wickstrom L, Hauser KE, Simmerling C. ff14SB: improving the accuracy of protein side chain and backbone parameters from ff99SB. *J Chem Theory Comput* (2015) 11(8):3696–713. doi:10.1021/acs.jctc.5b00255
37. Wang J, Wolf RM, Caldwell JW, Kollman PA, Case DA. Development and testing of a general AMBER force field. *J Comput Chem* (2004) 25:1157–74. doi:10.1002/jcc.20035
38. Hornung V, Rothenfusser S, Britsch S, Krug A, Jahrsdorfer B, Giese T, et al. Quantitative expression of toll-like receptor 1-10 mRNA cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. *J Immunol* (2002) 168:4531–7. doi:10.4049/jimmunol.168.9.4531
39. Yokota S, Ohnishi T, Muroi M, Tanamoto K, Fujii N, Amano K. Highly-purified *Helicobacter pylori* LPS preparations induce weak inflammatory reactions and utilize toll-like receptor 2 complex but not toll-like receptor 4 complex. *FEMS Immunol Med Microbiol* (2007) 51(1):140–8. doi:10.1111/j.1574-695X.2007.00288.x
40. Kagan JC, Su T, Horng T, Chow A, Akira S, Medzhitov R. TRAM couples endocytosis of toll-like receptor 4 to the induction of interferon-beta. *Nat Immunol* (2008) 9(4):361–8. doi:10.1038/ni1569
41. Piao W, Ru LW, Piepenbrink KH, Sundberg EJ, Vogel SN, Toshchakov VY. Recruitment of TLR adapter TRIF to TLR4 signaling complex is mediated by the second helical region of TRIF TIR domain. *Proc Natl Acad Sci U S A* (2013) 110(47):19036–41. doi:10.1073/pnas.1313575110
42. Thomas KE, Galligan CL, Newman RD, Fish EN, Vogel SN. Contribution of interferon-beta to the murine macrophage response to the toll-like receptor 4 agonist, lipopolysaccharide. *J Biol Chem* (2006) 281(41):31119–30. doi:10.1074/jbc.M604958200

43. Lin R, Heylbroeck C, Genin P, Pitha PM, Hiscott J. Essential role of interferon regulatory factor 3 in direct activation of RANTES chemokine transcription. *Mol Cell Biol* (1999) 19(2):959–66. doi:10.1128/MCB.19.2.959
44. Bandow K, Kusuyama J, Shamoto M, Kakimoto K, Ohnishi T, Matsuguchi T. LPS-induced chemokine expression in both MyD88-dependent and -independent manners is regulated by Cot/Tpl2-ERK axis in macrophages. *FEBS Lett* (2012) 586(10):1540–6. doi:10.1016/j.febslet.2012.04.018
45. Schromm AB, Brandenburg K, Loppnow H, Moran AP, Koch MH, Rietschel ET, et al. Biological activities of lipopolysaccharides are determined by the shape of their lipid A portion. *Eur J Biochem* (2000) 267(7):2008–13. doi:10.1046/j.1432-1327.2000.01204.x
46. Malgorzata-Miller G, Heinbockel L, Brandenburg K, van der Meer JW, Netea MG, Joosten LA. *Bartonella quintana* lipopolysaccharide (LPS): structure and characteristics of a potent TLR4 antagonist for in-vitro and in-vivo applications. *Sci Rep* (2016) 6:34221. doi:10.1038/srep34221
47. DeMarco ML, Woods RJ. From agonist to antagonist: structure and dynamics of innate immune glycoprotein MD-2 upon recognition of variably acylated bacterial endotoxins. *Mol Immunol* (2011) 49(1–2):124–33. doi:10.1016/j.molimm.2011.08.003
48. Miller SI, Ernst RK, Bader MW. LPS, TLR4 and infectious disease diversity. *Nat Rev Microbiol* (2005) 3:36–46. doi:10.1038/nrmicro1068
49. Steeghs L, Keestra AM, van Mourik A, Uronen-Hansson H, van der Ley P, Callard R, et al. Differential activation of human and mouse toll-like receptor 4 by the adjuvant candidate LpxL1 of *Neisseria meningitidis*. *Infect Immun* (2008) 76:3801–7. doi:10.1128/IAI.00005-08
50. Facchini FA, Zaffaroni L, Minotti A, Rapisarda S, Calabrese V, Forcella M, et al. Structure-activity relationship in monosaccharide-based toll-like receptor 4 (TLR4) antagonists. *J Med Chem* (2018) 61(7):2895–909. doi:10.1021/acs.jmedchem.7b01803
51. Sestito SE, Facchini FA, Morbioli I, Billod J-M, Martin-Santamaria S, Casnati A, et al. Amphiphilic guanidinocalixarenes inhibit lipopolysaccharide (LPS)- and lectin-stimulated toll-like receptor 4 (TLR4) signaling. *J Med Chem* (2017) 60(12):4882–92. doi:10.1021/acs.jmedchem.7b00095
52. Bryant CE, Spring DR, Gangloff M, Gay NJ. The molecular basis of the host response to lipopolysaccharide. *Nat Rev Microbiol* (2010) 8(1):8–14. doi:10.1038/nrmicro2266
53. Rietschel ET, Brade H, Holst O, Brade L, Muller-Loennies S, Mamat U, et al. Bacterial endotoxin: chemical constitution, biological recognition, host response, and immunological detoxification. *Curr Top Microbiol Immunol* (1996) 216:39–81.
54. Di Lorenzo F, Billod J-M, Martín-Santamaría S, Silipo A, Molinaro A. Gram negative extremophiles lipopolysaccharides: versatile molecules to survive in harsh habitats and promising source of inspiration for a new generation of endotoxin antagonists. *Eur J Org Chem* (2017) 28:4055–73. doi:10.1002/ejoc.201700113
55. Anwar MA, Panneerselvam S, Shah M, Choi S. Insights into the species-specific TLR4 signaling mechanism in response to *Rhodobacter sphaeroides* lipid A detection. *Sci Rep* (2015) 5:7657. doi:10.1038/srep07657
56. Rose JR, Christ WJ, Bristol JR, Kawata T, Rossignol DP. Agonistic and antagonistic activities of bacterially derived *Rhodobacter sphaeroides* lipid A: comparison with activities of synthetic material of the proposed structure and analogs. *Infect Immun* (1995) 63:833–9.
57. Christ WJ, McGuinness PD, Asano O, Wang Y, Mullarkey MA, Perez M, et al. Agonistic and antagonistic activities of bacterially derived *Rhodobacter sphaeroides* lipid A: comparison with activities of synthetic material of the proposed structure and analogs. *J Am Chem Soc* (1994) 116:3637–8. doi:10.1021/ja00087a075
58. Christ WJ, Asano O, Robidoux ALC, Perez M, Wang YA, Dubuc GR, et al. E5531, a pure endotoxin antagonist of high potency. *Science* (1995) 268:80–3. doi:10.1126/science.7701344
59. Nativel B, Couret D, Giraud P, Meilhac O, d'Hellencourt CL, Viranaïcken W, et al. *Porphyromonas gingivalis* lipopolysaccharides act exclusively through TLR4 with a resilience between mouse and human. *Sci Rep* (2017) 7(1):15789. doi:10.1038/s41598-017-16190-y
60. Manterola L, Moriyón I, Moreno E, Sola-Landa A, Weiss DS, Koch MH, et al. The lipopolysaccharide of *Brucella abortus* BvrS/BvrR mutants contains lipid A modifications and has higher affinity for bactericidal cationic peptides. *J Bacteriol* (2005) 187(16):5631–9. doi:10.1128/JB.187.16.5631-5639.2005
61. von Bargen K, Gorvel JP, Salcedo SP. Internal affairs: investigating the *Brucella* intracellular lifestyle. *FEMS Microbiol Rev* (2012) 36(3):533–62. doi:10.1111/j.1574-6976.2012.00334.x
62. Albers U, Tiaden A, Spirig T, Al Alam D, Goyert SM, Gangloff SC, et al. Expression of *Legionella pneumophila* paralogous lipid A biosynthesis genes under different growth conditions. *Microbiology* (2007) 153(Pt 11):3817–29. doi:10.1099/mic.0.2007/009829-0
63. Lück C, Helbig JH. Characterization of *Legionella* lipopolysaccharide. *Methods Mol Biol* (2013) 954:381–90. doi:10.1007/978-1-62703-161-5_24
64. Billod JM, Lacetera A, Guzmán-Caldentey J, Martín-Santamaría S. Computational approaches to toll-like receptor 4 modulation. *Molecules* (2016) 21(8):E994. doi:10.3390/molecules21080994
65. Tidswell M, Tillis W, Larosa SP, Lynn M, Wittek AE, Kao R, et al. Phase 2 trial of eritoran tetrasodium (E5564), a toll-like receptor 4 antagonist, in patients with severe sepsis. *Crit Care Med* (2010) 38(1):72–83. doi:10.1097/CCM.0b013e3181b07b78
66. Murakami D, Yamada H, Yajima T, Masuda A, Komune S, Yoshikai Y. Lipopolysaccharide inhalation exacerbates allergic airway inflammation by activating mast cells and promoting Th2 responses. *Clin Exp Allergy* (2007) 37:339–47. doi:10.1111/j.1365-2222.2006.02633.x
67. Jung YW, Schoeb TR, Weaver CT, Chaplin DD. Antigen and lipopolysaccharide play synergistic roles in the effector phase of airway inflammation in mice. *Am J Pathol* (2006) 168:1425–34. doi:10.2353/ajpath.2006.050986
68. Debarry J, Hanuszkiewicz A, Stein K, Holst O, Heine H. The allergy-protective properties of *Acinetobacter lwoffii* F78 are imparted by its lipopolysaccharide. *Allergy* (2010) 65(6):690–7. doi:10.1111/j.1398-9995.2009.02253.x

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 04 September 2018
doi: 10.3389/fimmu.2018.02006

Eicosanoid Control Over Antigen Presenting Cells in Asthma

Nincy Debeuf[1,2] *and Bart N. Lambrecht*[1,2,3]*

[1] *Laboratory of Immunoregulation, VIB-UGent Center for Inflammation Research, Ghent, Belgium,* [2] *Department of Internal Medicine, Ghent University, Ghent, Belgium,* [3] *Department of Pulmonary Medicine, Erasmus Medical Center, Rotterdam, Netherlands*

Asthma is a common lung disease affecting 300 million people worldwide. Allergic asthma is recognized as a prototypical Th2 disorder, orchestrated by an aberrant adaptive CD4+ T helper (Th2/Th17) cell immune response against airborne allergens, that leads to eosinophilic inflammation, reversible bronchoconstriction, and mucus overproduction. Other forms of asthma are controlled by an eosinophil-rich innate ILC2 response driven by epithelial damage, whereas in some patients with more neutrophilia, the disease is driven by Th17 cells. Dendritic cells (DCs) and macrophages are crucial regulators of type 2 immunity in asthma. Numerous lipid mediators including the eicosanoids prostaglandins and leukotrienes influence key functions of these cells, leading to either pro- or anti-inflammatory effects on disease outcome. In this review, we will discuss how eicosanoids affect the functions of DCs and macrophages in the asthmatic lung and how this leads to aberrant T cell differentiation that causes disease.

Keywords: eicosanoids, prostaglandins, leukotrienes, asthma, dendritic cells, macrophages

OPEN ACCESS

Edited by:
Otto Holst, Forschungszentrum Borstel (LG), Germany

Reviewed by:
Subhabrata Moitra, Instituto Salud Global Barcelona (ISGlobal), Spain
Marcus Peters, Ruhr-Universität Bochum, Germany

***Correspondence:**
Bart N. Lambrecht
bart.lambrecht@ugent.be

Specialty section:
This article was submitted to Inflammation, a section of the journal Frontiers in Immunology

Received: *02 July 2018*
Accepted: *14 August 2018*
Published: *04 September 2018*

Citation:
Debeuf N and Lambrecht BN (2018) Eicosanoid Control Over Antigen Presenting Cells in Asthma. Front. Immunol. 9:2006. doi: 10.3389/fimmu.2018.02006

PRIMER ON EICOSANOIDS, PROSTAGLANDINS AND LEUKOTRIENES

Eicosanoids are an important class of biologically active molecules, comprising prostanoids, leukotrienes (LTs) and lipoxins that have important pro- and anti-inflammatory effects in asthma. Under a variety of non-specific activation stimuli, such as pro-inflammatory mediators and other stress, the precursor molecule arachidonic acid (AA) is released from membrane phospholipids by cytosolic phospholipase A2. AA can be enzymatically converted either to prostanoids [prostaglandin (PG) and thromboxane] by COX enzymes or to LT and lipoxins by lipoxygenases (LOXs) (**Figure 1**).

Prostanoids The COX isozymes (constitutive COX-1 and inducible COX-2) catalyze the formation of PGG_2, which is then reduced to the intermediate PGH_2 through peroxidase activity. Various cell-specific PG synthases convert PGH_2 to biologically active products, such as PGE_2, PGI_2, PGD_2 and PGF_{2a} and thromboxane (TXA_2) (1). The differential expression and the distribution of these enzymes within cells present at sites of inflammation will determine the profile of prostanoid production. For instance, mast cells predominantly generate PGD_2 through their expression of hematopoietic PGD synthase (hPGDS). Through microsomal PGE_2 synthase (mPGES-1), PGE_2 is produced by virtually all lung cell types, but the most abundant sources are epithelial cells, fibroblasts, and macrophages (1). Prostanoids act in both paracrine and autocrine fashion through G protein-coupled receptors (GPCRs) on the surface of target cells. Interestingly, the distribution of prostanoid receptors on immune cells differs from the distribution of prostanoid-specific synthases. Prostanoid synthases are mainly expressed on innate immune

cells, whereas prostanoid receptors are expressed on both innate and adaptive immune system leukocytes (2). So, during inflammation, activated innate immune cells will produce prostanoids that act on lymphocytes in a paracrine manner and also modulate their own function in an autocrine way (3).

Leukotrienes are generated by LOX enzymes. The different LOX enzymes are named based on their positional specificity of AA oxygenation. For instance, 12-LOX oxygenates AA at carbon 12, resulting in 12-hydro(peroxy)eicosatetraenoic acid [12-H(P)ETE] (4). Since the human leukocyte-type 12-LOX is very similar to reticulocyte-type 15-LOX, these enzymes are often referred to in the literature as 12/15-LOXs (5). Furthermore, mice do not express 15-LOX and only express the leukocyte-derived 12-LOX. Because murine 12-LOX is also able to generate 15-H(P)ETE, the enzyme is often designated as 12/15 LOX as well (6).

5-lipoxygenase (5-LOX) generates the leukotriene LTA_4, an unstable intermediate, which is converted to the chemoattractant LTB_4 or to nonchemotactic LTC_4 by the cytosolic LTA_4 hydrolase enzyme or leukotriene C4 synthase (LTC_4S) respectively. LTC_4 is exported to the extracellular space and is further converted to the unstable LTD_4 and subsequently to the stable end-metabolite LTE_4 (7). LTC_4, LTD_4 and LTE_4 belong to the so-called cysteinyl leukotrienes, due to the presence of the amino acid cysteine in their structure. There are at least three different cysteinyl leukotriene receptors (CysLTR1, CysLTR2, and CysLTR3). LTE_4 preferably binds CysLTR3 (8), whereas LTC_4 binds CysLTR2 and LTD_4 binds both CysLTR1 and CysLTR2 (9, 10).

Leukotrienes are predominantly produced by leukocytes, hence their name. However, the specific profile of LTs produced depends on the cell type. Neutrophils produce exclusively LTB_4, whereas mast cells, basophils and eosinophils mainly produce cysLTs. Macrophages and DCs synthesize both LTB_4 and cysLTs (11).

Lipoxins (LXA_4 and LXB_4) are short-lived eicosanoids that are derived from arachidonic acid through sequential activity of 5-LOX and 12/15-LOX. 15-LOX is a key enzyme for lipoxin generation in the human lung and is expressed by many cells during inflammation, including macrophages, eosinophils and bronchial epithelial cells (12–14).

EICOSANOIDS HAVE MULTIPLE EFFECTS IN ALLERGIC ASTHMA

Asthma is a chronic inflammatory disease of the airways, characterized by reversible bronchoconstriction, airway remodeling and mucus production. Most childhood-onset asthma and half of the adult-onset asthma cases are allergic, identified by a positive skin prick test or the detection of serum IgE antibodies against common antigens, such as plant and tree pollen, animal dander, house dust mites (HDM) and fungal spores. Virtually all cell types relevant to Th2 pathology such as Th2 cells, ILC2s, mast cells, basophils, epithelial cells, smooth muscle cells and fibroblasts generate LT and/or PG mediators, and/or express receptors for those eicosanoids (**Figure 2**). Among prostanoids, PGD_2 released from mast cells, has long been implicated in allergic diseases (15). PGD_2 is known to have chemotactic effects on eosinophils, basophils, Th2 lymphocytes and ILC2s acting via the DP2/CRTh2 receptor (16, 17) and in this way contributes to airway hyperresponsiveness, IgE and cytokine secretion (18–20). PGD_2 levels and the number of CRTH2+ cells are increased in bronchoalveolar lavage (BAL) fluids from severe asthmatics compared to those with milder disease (21). Several CRTH2 antagonists have shown encouraging results in clinical trials for asthma, further supporting for the role of PGD_2 in allergic diseases and its potential as a therapeutic target (22).

Although cyclooxygenase and its products, PGs, have been traditionally linked to all four cardinal signs of inflammation (redness, swelling, heat, pain), prostanoids may also have an inhibitory role on inflammatory cells. This discrepancy can be explained by the fact that typical inflammation hallmarks are the result of actions on microvasculature, hypothalamus and nerves, rather than on immune cells. In mouse models of asthma, PGs have pleiotropic effects. PGI_2 can abolish asthma development by inhibition of DC activation and Th2 cell migration (23–25), whereas PGE_2 can reduce mast cell secretory responses (26–29) and chemotaxis of eosinophils (30). Furthermore, both PGI_2 and PGE_2 can inhibit cytokine release of both Th1 and Th2 CD4 T cells and macrophages (31, 32). Treg differentiation and function is also promoted by PGE_2 (33, 34).

Prostanoids are also able to inhibit airway remodeling and mucus secretion in asthma models *in vivo* (35–37). It has been shown that PGE_2 induces fibroblast apoptosis (38), abolishes myofibroblast differentiation (39) and inhibits proliferation of airway smooth muscle cells (40).

In asthma patients, inhalation of exogenous PGE_2 or its analogs results in bronchodilatation and protection against early- and late-phase bronchoconstriction induced by various asthma triggers (41–43). Despite the benefits of inhaled PGE_2, it has also been reported that prostanoids can induce irritancy of the upper airway resulting in a reflex cough. However, this can be overcome by treatment with a receptor-selective agonist, as cough is exclusively mediated via the EP3 receptor (44).

In contrary to the bronchodilatory properties of the prostaglandin PGE_2, CysLTs are an important cause of allergen-induced bronchoconstriction (45). Indeed, treatment with Cysteinyl LT receptor 1 antagonists (LTRAs) attenuates allergen-induced increases in airway hyperresponsiveness (46, 47). Furthermore LTRAs partially attenuate allergen-induced airway eosinophilia (47, 48), demonstrating a more extensive role for LTs in asthma. Indeed, CysLTs that are also released from mast cells, particularly LTE_4, can cause eosinophil chemotaxis in allergic asthmatics (49). Interestingly, CysLT levels are also increased in BAL fluid (50) and in urine after allergen challenge (51). Currently, LTRAs (such as montelukast) are clinically available. Although these drugs are superior to placebo at decreasing asthmatic symptoms and exacerbations, LTRAs are not recommended as first line therapy for asthma. The reason for this is that they are generally inferior to

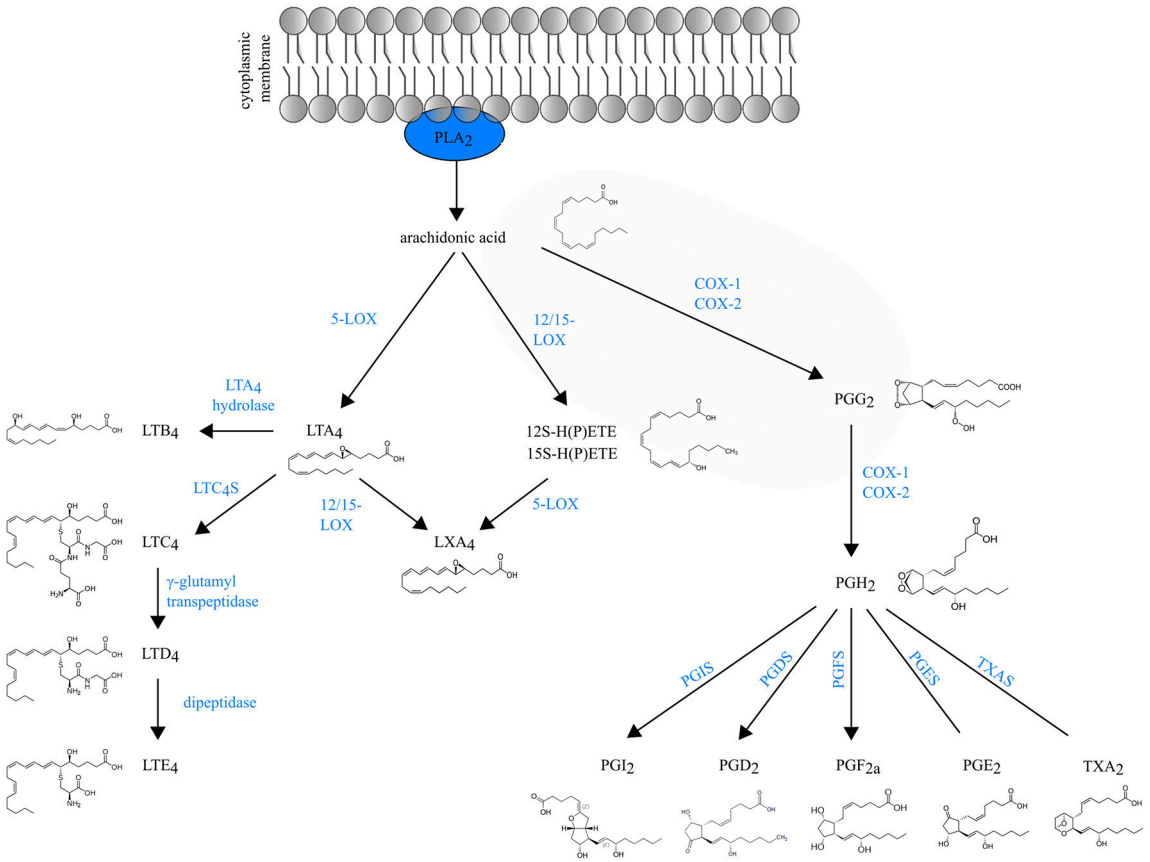


FIGURE 1 | Schematic overview of eicosanoid biosynthesis. Arachidonic acid that is released from membrane phospholipids by cytosolic phospholipase A2 (PLA_2), can be enzymatically converted either to prostaglandins (PG) and thromboxane (TXA_2) by COX enzymes or to LT and lipoxins (LXA_4) by lipoxygenases (LOXs).

inhaled corticosteroids in anti-inflammatory and clinical effects[1]. Furthermore, about one third of the asthma patients does not respond to LTRAs (52).

Another type of leukotriene, LTB_4, through its actions on the BLT1 receptor, is an activator and chemoattractant for different cell types such as T cells (53, 54) and DCs (55). OVA-induced allergic inflammation was completely abolished in BLT1 deficient mice, demonstrating the importance of BLT1 and its ligand LTB_4 in the development of allergic airway inflammation (56).

Lipoxins have a pro-resolution role in allergic airway inflammation. In severe asthmatics, blood LXA_4 levels and leukocyte LXA_4 generation are reduced compared to those with milder disease (57–59). In a mouse model of asthma, administration of a stable analog of LXA_4 resulted in a diminished airway hyperresponsiveness and pulmonary inflammation (60, 61). Similar results were obtained with resolvins and protectins. Those mediators are also generated by LOX enzymes, but are derived from omega-3 polyunsaturated fatty acids instead of the substrate arachidonic acid (62–64).

Finally, absence of all eicosanoids impairs the induction of a Th2 response and reduces airway inflammation. This has been shown with mice lacking group V secretory phospholipase A2 (sPLA2), which is the enzyme that releases AA from membrane lipids and catalyzes the first step of eicosanoid generation. Deletion of sPLA2 attenuates cell migration and airway hyperresponsiveness, whereas sPLA2 overexpression is associated with severe asthma (65–68). An impaired antigen capture activity and maturation of DCs is responsible for the inhibition of asthma development in $sPLA2^{-/-}$ mice (69).

CURRENT INSIGHTS IN ALLERGIC ASTHMA PATHOGENESIS: A CENTRAL ROLE FOR DENDRITIC CELLS

In allergic asthma, airway DCs take up allergens across the epithelial barrier and subsequently activate Th2 immunity in the draining lymph nodes, leading to IgE responses and to Th2 effector cells that control eosinophilic airway inflammation,

[1]National Institutes of Health; National Heart, Lung, and Blood. National Asthma Education and Prevention Program Institute, Expert panel report 3: guidelines for the diagnosis and management of asthma. No. 07–4051 Available from: http://www.nhlbi.nih.gov/guidelines/asthma/asthgdln.htm 2007.

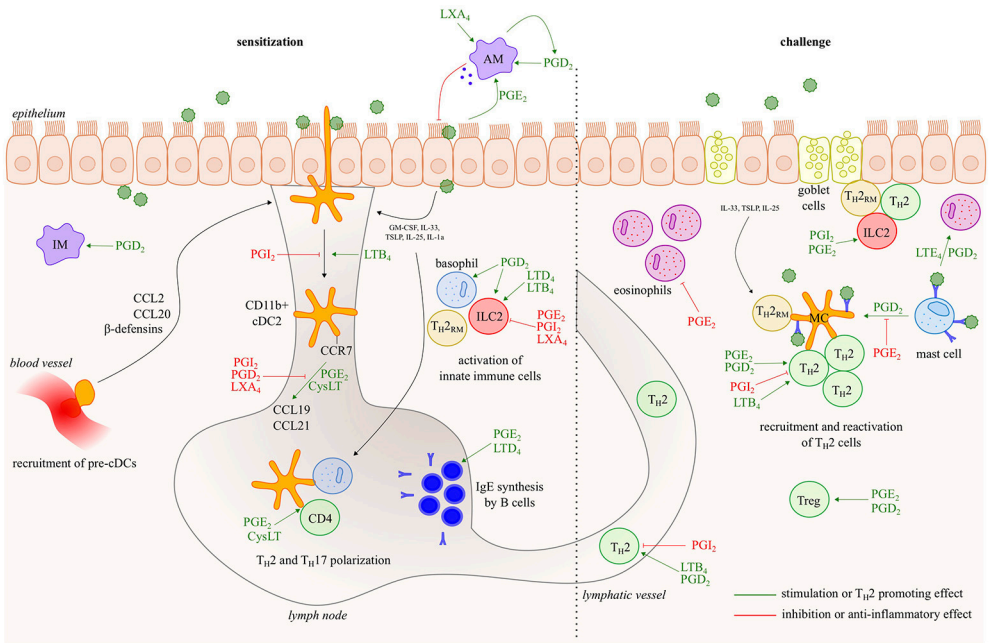


FIGURE 2 | Eicosanoids have multiple effects in allergic asthma. In response to allergens and/or instructive cytokines by epithelial cells and innate immune cells, dendritic cells activate Th2 immunity in the draining lymph nodes, leading to IgE responses and to Th2 effector cells that control eosinophilic airway inflammation, goblet cell metaplasia and bronchial hyperreactivity upon return to the lung. Virtually all cell types relevant to Th2 pathology such as Th2 cells, ILC2s, mast cells, basophils and epithelial cells, generate leukotriene/prostaglandin mediators, and/or express receptors for those eicosanoids. The figure represents a schematic overview of eicosanoid functions described in this review.

goblet cell metaplasia and bronchial hyperreactivity upon return to the lung (70, 71). The central role for DCs in the development of allergic asthma has been demonstrated in numerous studies. Adoptive transfer of GM-CSF-cultured bone marrow-derived DCs (BMDCs) or splenic DCs that were pulsed with ovalbumin (OVA) antigen *in vitro* can sensitize mice, leading to a Th2 response and eosinophilic inflammation upon challenges with OVA aerosol (72, 73). Likewise, DCs originating from the lungs of allergen-exposed mice are also able to induce sensitization when transferred to naive recipients (74, 75). This holds also true for chronic asthma models as repeated DC injection into the lung induces irreversible airway remodeling, characterized by subepithelial collagen deposition and increased peribronchial airway smooth muscle volume (76).

In addition to these studies demonstrating that DCs are sufficient for induction of Th2 immunity in the lung, DCs are also required for inducing a Th2 response to allergens, even in very young mice before weaning (77). Depletion of lung DCs in CD11c-DTR transgenic mice during the first exposure to the inhaled HDM allergen impeded the development of lung eosinophilia and Th2 cytokine production (74). Likewise, DCs are also required for optimal Th2 immunity against other allergens, such as papain and helminths (78, 79).

Beside the crucial role of DCs in inducing Th2 immunity in naïve animals, DCs have also a non-redundant role during the secondary immune response (76, 80). During the challenge phase, DCs are closely located to antigen-specific T cells around the airways and large blood vessels (81). Here, they might secrete chemokines to attract effector T cells or they might restimulate resident memory T cells by providing costimulatory molecules (75, 82).

Murine lungs in steady state contain three major subsets of DCs with specific phenotype and functions; pDCs, IRF8-dependent XCR1+ CD103+ cDC1s and IRF4-dependent CD11b+ SIRPα+ cDC2s. However, during inflammation monocyte-derived DCs (MCs) emerge, coming from monocytes that migrate to the local tissue and upregulate the expression of CD11c and MHC-II (75, 83). They can be distinguished from CD11b+ cDCs by the expression of the Fc receptors CD64 and MAR1 (75). Various studies have shown that CD11b+ cDC2s are the responsible DC subtype for Th2/17 induction upon allergen challenge (75, 84–86). MCs rather play a role during the effector of the immune response, by interacting with effector Th2 cells that migrate back to the lung or with resident-memory T cells (87). In contrast to CD11b+ cDC2s, CD103+ cDC1s play a redundant role in the HDM-driven asthma model (75). There is even literature suggesting that cDC1s induce a tolerogenic response to inhaled allergens (88–90). An immunoregulatory role has also been described for pDCs. Indeed, it has been shown that pDCs in the lung are essential to induce inhalation tolerance to harmless antigens like OVA (91, 92). Furthermore, depletion of pDCs during sensitization or challenge to OVA or HDM allergen might exacerbate inflammation, as immunoregulatory regulatory

T cells fail to function properly in the absence of pDCs (91, 93, 94).

Although DCs express PRRs and can sense the environment directly, the epithelium has been shown to be equally important in activating DCs in response to allergens (95). As this is beyond the scope of this review, we refer to Hammad and Lambrecht for a recent review describing the role of epithelial cytokines in the activation of DCs during allergic inflammation (96). In brief, DCs get activated by epithelial cytokines like IL-33, GM-CSF, IL-1a, IL-25, and thymic stromal lymphopoietin (TSLP). The same cytokines also activate ILC2s, basophils and Th2 effector cells to become cytokine producing cells and contribute to the initiation of a Th2 response (70, 71, 97). The release of epithelial cytokines is elicited by environmental stimuli of asthma, such as HDM, viruses, diesel particles and cigarette smoke. On the other hand, protective environments, such as farm dust or lipopolysaccharide exposure, have the potential to suppress this cytokine release and DC activation (98).

EICOSANOIDS AFFECT THE MIGRATION OF DENDRITIC CELLS

The control of DC migration is pivotal for the initiation of cellular immune responses. Upon activation by inflammatory stimuli, DCs upregulate the chemokine receptor CCR7 and home to lymphoid organs, where the CCR7 ligands CCL19 and CCL21 are expressed. This migratory capacity of DCs requires environmental instruction by PGE_2. PGE_2 has no effect on the expression of CCR7 on DCs, but couples CCR7 expression to signal transduction pathways such as activation of cAMP-dependent protein kinase A (PKA) and Rho Kinase (99). These signals allow the DCs to start migration, among other by inducing a rapid disassembly of podosomes (100). Surprisingly, PGE_2 was only required at early time points of maturation to enable DC chemotaxis, whereas PGE_2 addition has no effect during terminal maturation. Mouse DCs exclusively rely on EP4 receptor triggering for migration, whereas human MCs require a signal mediated by EP2 or EP4 either alone or in combination (101, 102).

In contrary to PGE_2, PGD_2, and PGI_2 inhibit the maturation and migration of DCs. In the skin, Angeli and colleagues showed that parasite-derived PGD_2 inhibits the migration of epidermal Langerhans cells to the skin draining lymph nodes and affects the subsequent cutaneous inflammatory reaction (103). Similarly, intratracheal instillation of FITC-OVA together with PGD_2 inhibits the migration of FITC+ lung DC to draining LNs. Activation of the DP1 receptor was responsible for this inhibition (104). DP1 activation also lowers the expression of costimulatory molecules on DCs and enhances the induction of Foxp3+ Treg cells, resulting in an abolished asthma phenotype (34). Inhalation of iloprost, a stable PGI_2 analog, also suppressed the cardinal features of asthma by interfering with the function of lung myeloid DC. Furthermore, iloprost-treated DCs no longer induced Th2 differentiation from naive T cells or boosted effector cytokine production in primed Th2 cells, showing that the effect of iloprost was DC intrinsic (23).

CysLT enhance the migration of DCs. Indeed, DCs lacking the LTC4 transporter multidrug resistance-associated protein 1 (MRP1) failed to migrate to the lymph nodes, whereas exogenous LTC_4 or LTD_4 could restore this migration. However, these CysLTs only promoted optimal chemotaxis to the chemokine CCL19, but not to other related chemokines (105). On the other hand, lipoxins were able to inhibit DC migration (106).

EICOSANOID SIGNALING IN DCS MODULATES INSTRUCTION OF T CELL DIFFERENTIATION

Upon DC-T cell encounter, DCs produce cytokines that drive Th differentiation. The secretion pattern of these cytokines, and thus the Th1/Th2 balance can be modulated by a variety of biologically active mediators synthesized by innate and adaptive immune cells. Eicosanoids such as PGE_2 exert a great impact on this regulation. For instance, the ratio of PGE_2 and IL-12, both produced by APCs, may control the balance between Th1 and Th2 immunity (107). Basically, it has been shown that PGE_2 is a potent inhibitor of IL-12 production (108) and in this way favors a Th2 response (109–112). PGE_2 also inhibits the secretion of TNF-α from murine DCs (113, 114). The inhibitory role of PGE_2 on DC cytokine secretion can also be indirect by inducing IL-10 secretion (108, 113, 115). Due to its inhibitory effect on IL-12, PGE_2 also indirectly inhibits IFN-γ secretion by T cells and NK cells (110, 116).

Beside the Th2 inducing role for PGE_2, it has also been reported that PGE_2-treated DCs can induce Th1 and Th17 responses. Adding PGE_2 together with TNF-α to human BMDCs stimulates IL-12 production by DCs, favoring a Th1 response (117–121). PGE_2 also stimulates IL-23 production by cultured BMDCs and promotes in this way Th17 differentiation (122, 123).

Prostanoids can also directly modify production of Th cytokines from polarized T cells. PGE_2 can favor Th2 immunity by inhibiting IL-2 and IFN-γ production by Th1 cells, but not the production of IL-4 by Th2 cells (124, 125). However, in a mouse model of asthma, PGE_2 has also been shown to inhibit Th2 responses via direct effects on the EP2 receptor on T cells (126). Furthermore, PGE_2 regulates Th17 cell differentiation and cytokine secretion directly through EP2/EP4 receptor signaling on T cells (127). Via DP1, PGD_2 can block the expression of the Th1 cytokine IFN-γ. Furthermore, Th2 cytokine secretion is increased through CRTH2 signaling (128). On the other hand, PGI_2 can directly inhibit production of Th2 cytokines from Th2 polarized mouse splenic CD4+ cells (32, 129), thus directly exhibiting lower levels of Th2 response.

Less is known about the role of leukotrienes on T cell polarization. Machida *et al*. reported that *in vitro* treatment with LTRAs modifies the cytokine profile of DCs (130). By *in vivo* administration of LTRAs, Okunishi and colleagues showed that LTs promote DC antigen presentation and both Th1 and Th2 polarizing cytokine secretion (131).

Furthermore, using $LTC_4^{-/-}$ and $CysLTR1^{-/-}$ mice, it has been demonstrated that leukotrienes are crucial for the initiation

of a Th2 response upon HDM-dependent Dectin-2 activation on DCs (132). Through CysLTR1, LTD_4 can induce IL-4 secretion by ILC2s, contributing to Th2 polarization as well (133). CysLTR1 can also be up-regulated in activated CD4+ T cells themselves and can mediate their chemotaxis to LTD_4, but whether cysLTs exert a direct effect on cytokine production by CD4+ T cells remains unclear (134). This is different from the leukotriene LTB4, which increases cytokine production by T cells (135), but does not affect antigen presentation and cytokine production by DCs (55). Strikingly, mice deficient in CysLTR2 or adoptive transfer of DCs lacking CysLTR2 developed markedly enhanced Th2 immunity to HDM. In fact, CysLTR2 negatively regulates cell surface expression and receptor signaling of DCs (136). Thus, the biologic activity of CysLTs can be tightly regulated by competition between the different expressed CysLT receptors.

12/15-LOX enzymes, required for lipoxin synthesis, are also involved in the modulation of Th2 cytokine secretion. In response to IL-13, DCs secrete the lectin Ym1/2 that might interact with 12/15-LOX in or at the surface of T cells. 12/15-LOX generates 12-HETE that has been shown to reduce Th2 cytokine secretion both *in vivo* and *in vitro*. Furthermore, 12-HETE attenuated airway eosinophilia in an OVA-induced allergic asthma model. However, DC-secreted Ym1/2 was able to decrease the expression of 12-HETE, suggesting that the asthma-promoting effects of Ym1/2 might be explained by inhibiting 12/15-LOX on T cells (137).

EICOSANOIDS ALSO AFFECT ANTIGEN-PRESENTING B CELLS AND ILC2S

The most described function of B cells is their production of antigen-specific immunoglobulins. However, in addition to antibody production, activated B cells also play a role as accessory antigen-presenting cells. Although they are not as potent as DCs in priming naïve T cells, they are abundantly present in T-cell inductive sites, express costimulatory molecules and produce cytokines that activate DCs and naïve T cells (138–141). Their antigen-presenting and Th2-promoting effects have also been demonstrated in murine asthma models, with a particular role during secondary challenge and when the antigen dose is limiting (142).

Numerous studies have shown that eicosanoids are required for both the development and function of B lymphocytes (143–147). PGE_2 is necessary for IgE production both *in vitro* and *in vivo*, by affecting IgE class switching (145, 148–151). Furthermore, PGE_2 has been demonstrated to regulate B cell proliferation (152). Interestingly, PGE_2 is also able to lower MHCII expression on B cells (148), but whether this affects antigen presentation is still unclear. Leukotrienes, in particular LTD_4, can enhance immunoglobulin production as well (153). In contrast, lipoxins have the opposite effect as 12/15-LOX deficiency protects mice from allergic airway inflammation by increasing secretory IgA levels (147).

Strikingly, ILC2s have also been shown to present antigen (154, 155). In response to the parasitic worm *Nippostrongylus brasiliensis*, MHC class II expression on ILC2s was required for the induction of an efficient Th2 response. ILC2s express the costimulatory receptors CD80 and CD86, acquire and process antigen and interact with antigen-specific T cells. During this interaction, T cell-derived IL-2 promotes ILC2 proliferation and IL-13 production (155) and this can be affected by eicosanoids. PGD_2 and CysLTs stimulate Th2 cytokine production from ILC2s (133, 156, 157), whereas other lipid mediators have suppressive roles on ILC2 function. The pro-resolving mediator LXA_4 could inhibit ILC2 activation (156) and both PGE_2 and PGI_2 were able to attenuate ILC2 proliferation, Th2 cytokine generation and resulting type 2 immune response (158, 159).

EICOSANOIDS MODULATE THE TOLEROGENIC ROLE OF MACROPHAGES IN THE ALLERGIC LUNG

Lung macrophages can be divided into alveolar macrophages (AMs) and interstitial macrophages (IMs). AMs are most abundantly present and are situated in the alveolar lumen, while IMs are located inside the lung interstitium. During inflammation, a third population emerges, as monocyte-derived macrophages infiltrate the alveolar and interstitial areas. Macrophages express different eicosanoid receptors, such as the receptors for PGE_2 and PGD_2. Furthermore, macrophages produce both prostaglandins and leukotrienes themselves, allowing autocrine regulation (160).

AMs are sessile, long-lived, and self-renewing cells that derive from fetal monocytes under the influence of GM-CSF (161–164). Several studies have clearly demonstrated that resident AMs induce a tolerogenic response to inhaled antigens (164–171). Use of liposomal clodronate to deplete resident AMs in an OVA or HDM-induced asthma model, favored a Th2 response and subsequently resulted in increased BAL eosinophilia and inflammatory cytokine levels (167, 168). One possible mechanism for this inhibitory role of macrophages is the secretion of SOCS1 and SOCS3 in exosomes and microparticles. The uptake of these particles by alveolar epithelial cells inhibits their activation in a JAK/STAT-dependent way (169). PGE_2 is a major epithelium-derived factor mediating SOCS secretion (170) and in this way inhibiting the development of allergic lung inflammation (171). Indeed, in a HDM-dependent asthma model, adoptive transfer of PGE_2-treated macrophages led to a reduction in eosinophilia in the allergic lung (171).

Pulmonary inflammation was also reduced if macrophages lacked group V sPLA2, which is the enzyme releasing AA from membrane lipids and is required for both PG and LT synthesis (172). Those macrophages generated less PGE_2, resulting in a diminished transglutaminase activity of M2 macrophages (173). Furthermore, by activating the EP4 receptor on macrophages, PGE_2 inhibits TNF-α and IL-12 cytokine secretion (31). The ability of lung macrophages to prevent Th2 induction in response to inhaled allergens has also been demonstrated in rats. The replacement of AM of sensitized animals by AM from naive animals completely abolished Th2 polarization by inhibition of DC allergen capture and migration to the lymph nodes (174).

Although PGE_2 suppresses type 2 inflammation in most settings, a recent study demonstrated that PGE_2 also has pro-inflammatory effects in murine macrophages. Mice lacking microsomal PGE_2 synthase 1 (mPGE1) had an attenuated asthma phenotype compared to wild-type controls in response to repetitive inhalation challenges with an extract from the allergenic mold *Alternaria alternata*, which could be explained by a diminished IL-33 production by murine macrophages (175).

The pro-inflammatory prostanoid PGD_2 binds both the DP1 and DP2 receptor on lung macrophages. DP signaling enhances migration and TNF-α secretion of both alveolar and interstitial macrophages. Furthermore, PGD_2 also induces KC secretion from macrophages, resulting in neutrophil recruitment in the lung and this neutrophilia could be abolished by macrophage depletion (176). Interestingly, PGD_2 synthesis by macrophages is also involved in the enhancement of airway inflammation by virus infections. Respiratory infections with RNA viruses, such as rhinovirus or respiratory syncytial virus (RSV), are associated with asthmatic exacerbations (177). To study the mechanism behind this association, Shiraishi and colleagues administered poly I:C, a synthetic dsRNA, intratracheally in OVA-sensitized rats. Those rats developed an exacerbated asthma phenotype and had elevated PGD_2 synthesis in the lung, particularly in AMs. CRTH2-deficient animals did not exhibit a dsRNA-induced increase in eosinophil accumulation, demonstrating the necessary role for PGD_2 in dsRNA-induced enhancement of airway inflammation (178).

RSV infection of mice deficient in 5-LOX, an enzyme required for lipoxin synthesis, resulted in stronger lung pathology compared to wildtype mice, due to a lack of alternatively activated macrophages (179, 180). Treatment with LXA_4 partially restored this, supporting a pro-resolution role for lipoxins in viral respiratory tract infections (180).

Just as described for DCs, AMs produce leukotrienes in response to HDM-driven Dectin-2 activation. Both an inhibitor of LT production and Dectin-2 blockade could prevent the development of bronchial hyperreactivity and airway inflammation, demonstrating the required role for Dectin-2 dependent leukotriene production in the initiation of allergic airway inflammation (181).

SUMMARY

Allergic asthma is a chronic lung disease, driven by a prototypical Th2 response against airborne allergens. Dendritic cells (CD11b + cDC2s) are indispensable and sufficient for the development of allergic asthma, whereas macrophages have merely a tolerogenic role. Eicosanoids, leukotrienes and prostaglandins, influence key functions of these cells. However, given the diverse spectrum of eicosanoids and given the cell-type dependent expression profile of eicosanoid receptors, it is not surprising that the effects of PG/LT can be very distinct depending on the inflammatory context. A particular eicosanoid can have a pro-inflammatory effect on a certain cell type, whereas it can act anti-inflammatory on another. Furthermore, one particular cell type will be exposed to both pro-inflammatory and anti-inflammatory eicosanoids and the balance between those will determine the cellular outcome. Leukotriene receptor antagonists are already in clinical use for the treatment of asthma. In addition, given the multiple roles of prostaglandins in the pathogenesis of asthma, PG a-/antagonists may also have a promising therapeutic effect.

AUTHOR CONTRIBUTIONS

ND wrote the first draft of the manuscript. BL edited the manuscript. Both authors contributed to manuscript revision, read and approved the submitted version.

FUNDING

ND is supported by a Fonds Wetenschappelijk Onderzoek Vlaanderen (FWO) grant (11Y8417N). BL is supported by an European Research Council (ERC)-Consolidator grant (EU789384) and Excellence of Science (EOS) research grant (project 30565447).

REFERENCES

1. Funk CD. Prostaglandins and leukotrienes: advances in eicosanoid biology. *Science* (2001) 294:1871–5. doi: 10.1126/science.294.5548.1871
2. Tilley SL, Coffman TM, Koller BH. Mixed messages: modulation of inflammation and immune responses by prostaglandins and thromboxanes. *J Clin Invest.* (2001) 108:15–23. doi: 10.1172/JCI2001 13416
3. Harizi H, Gualde N. The impact of eicosanoids on the crosstalk between innate and adaptive immunity: the key roles of dendritic cells. *Tissue Antigens* (2005) 65:507–14. doi: 10.1111/j.1399-0039.2005.00394.x
4. Brash AR. Lipoxygenases: occurrence, functions, catalysis, and acquisition of substrate. *J Biol Chem.* (1999) 274:23679–82. doi: 10.1074/jbc.274.34.23679
5. Kühn H, Walther M, Kuban RJ. Mammalian arachidonate 15-lipoxygenases structure, function, and biological implications. *Prostaglandins Other Lipid Mediat.* (2002) 68–9:263–290. doi: 10.1016/S0090-6980(02)00035-7
6. Chen XS, Kurre U, Jenkins NA, Copeland NG, Funk CD. cDNA cloning, expression, mutagenesis of C-terminal isoleucine, genomic structure, and chromosomal localizations of murine 12-lipoxygenases. *J Biol Chem.* (1994) 269:13979–87.
7. Samuchiwal SK, Boyce JA. Role of lipid mediators and control of lymphocyte responses in type 2 immunopathology. *J Allergy Clin Immunol.* (2018) 141:1182−90. doi: 10.1016/j.jaci.2018.02.006
8. Kanaoka Y, Maekawa A, Austen KF. Identification of GPR99 protein as a potential third cysteinyl leukotriene receptor with a preference for leukotriene E4 ligand. *J Biol Chem.* (2013) 288:10967–72. doi: 10.1074/jbc.C113.453704
9. Nothacker HP, Wang Z, Zhu Y, Reinscheid RK, Lin SH, Civelli O. Molecular cloning and characterization of a second human cysteinyl leukotriene receptor: discovery of a subtype selective agonist. *Mol Pharmacol.* (2000) 58:1601–8. doi: 10.1124/mol.58.6.1601
10. Lynch KR, O'Neill GP, Liu Q, Im DS, Sawyer N, Metters KM, et al. Characterization of the human cysteinyl leukotriene CysLT1 receptor. *Nature* (1999) 399:789–93. doi: 10.1038/21658
11. Peters-Golden M, Henderson WR Jr. Leukotrienes. *N Engl J Med.* (2007) 357:1841–54. doi: 10.1056/NEJMra071371
12. Hunter JA, Finkbeiner WE, Nadel JA, Goetzl EJ, Holtzman MJ. Predominant generation of 15-lipoxygenase metabolites of arachidonic acid by epithelial cells from human trachea. *Proc Natl Acad Sci USA.* (1985) 82:4633–7. doi: 10.1073/pnas.82.14.4633

13. Levy BD, Romano M, Chapman HA, Reilly JJ, Drazen J, Serhan CN. Human alveolar macrophages have 15-lipoxygenase and generate 15(S)-hydroxy-5,8,11-cis-13-trans-eicosatetraenoic acid and lipoxins. *J Clin Invest.* (1993) 92:1572–9. doi: 10.1172/JCI116738
14. Serhan CN, Hirsch U, Palmblad J, Samuelsson B. Formation of lipoxin A by granulocytes from eosinophilic donors. *FEBS Lett.* (1987) 217:242–6. doi: 10.1016/0014-5793(87)80671-3
15. Hardy CC, Robinson C, Tattersfield AE, Holgate ST. The bronchoconstrictor effect of inhaled prostaglandin D2 in normal and asthmatic men. *N Engl J Med.* (1984) 311:209–13. doi: 10.1056/NEJM198407263110401
16. Hirai H, Tanaka K, Yoshie O, Ogawa K, Kenmotsu K, Takamori Y, et al. Prostaglandin D2 selectively induces chemotaxis in T helper type 2 cells, eosinophils, and basophils via seven-transmembrane receptor CRTH2. *J Exp Med.* (2001) 193:255–61. doi: 10.1084/jem.193.2.255
17. Nagata K, Hirai H, Tanaka K, Ogawa K, Aso T, Sugamura K, et al. CRTH2, an orphan receptor of T-helper-2-cells, is expressed on basophils and eosinophils and responds to mast cell-derived factor(s). *FEBS Lett.* (1999) 459:195–9. doi: 10.1016/S0014-5793(99)01251-X
18. Pettipher R, Whittaker M. Update on the development of antagonists of chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2). From lead optimization to clinical proof-of-concept in asthma and allergic rhinitis. *J Med Chem.* (2012) 55:2915–31. doi: 10.1021/jm2013997
19. Xue L, Salimi M, Panse I, Mjösberg JM, McKenzie ANJ, Spits H, et al. Prostaglandin D2 activates group 2 innate lymphoid cells through chemoattractant receptor-homologous molecule expressed on TH2 cells. *J Allergy Clin Immunol.* (2014) 133:1184–94. doi: 10.1016/j.jaci.2013.10.056
20. Chang JE, Doherty TA, Baum R, Broide D. Prostaglandin D2 regulates human type 2 innate lymphoid cell chemotaxis. *J Allergy Clin Immunol.* (2014) 133:899–901.e3. doi: 10.1016/j.jaci.2013.09.020
21. Fajt ML, Gelhaus SL, Freeman B, Uvalle CE, Trudeau JB, Holguin F, et al. Prostaglandin D2 pathway upregulation: relation to asthma severity, control, and TH2 inflammation. *J Allergy Clin Immunol.* (2013) 131:1504–12. doi: 10.1016/j.jaci.2013.01.035
22. George L, Brightling CE. Eosinophilic airway inflammation: role in asthma and chronic obstructive pulmonary disease. *Ther Adv Chronic Dis.* (2016) 7:34–51. doi: 10.1177/2040622315609251
23. Idzko M, Hammad H, van Nimwegen M, Kool M, Vos N, Hoogsteden HC, et al. Inhaled iloprost suppresses the cardinal features of asthma via inhibition of airway dendritic cell function. *J Clin Invest.* (2007) 117:464–72. doi: 10.1172/JCI28949
24. Nagao K, Tanaka H, Komai M, Masuda T, Narumiya S, Nagai H. Role of prostaglandin I2 in airway remodeling induced by repeated allergen challenge in mice. *Am J Respir Cell Mol Biol.* (2003) 29:314–20. doi: 10.1165/rcmb.2003-0035OC
25. Jaffar Z, Ferrini ME, Buford MC, FitzGerald GA, Roberts K. Prostaglandin I2-IP signaling blocks allergic pulmonary inflammation by preventing recruitment of CD4+ Th2 cells into the airways in a mouse model of asthma. *J Immunol.* (2007) 179:6193–203. doi: 10.4049/jimmunol.179.9.6193
26. Herrerias A, Torres R, Serra M, Marco A, Roca-Ferrer J, Picado C, et al. Subcutaneous prostaglandin E(2) restrains airway mast cell activity *in vivo* and reduces lung eosinophilia and Th(2) cytokine overproduction in house dust mite-sensitive mice. *Int Arch Allergy Immunol.* (2009) 149:323–32. doi: 10.1159/000205578
27. Torres R, Pérez M, Marco A, Picado C, de Mora F. A Cyclooxygenase-2 selective inhibitor worsens respiratory function and enhances mast cell activity in ovalbumin-sensitized mice. *Arch Bronconeumol. (English Edition)* (2009) 45:162–7. doi: 10.1016/j.arbres.2008.04.007
28. Torres R, Herrerias A, Serra-Pagès M, Marco A, Plaza J, Costa-Farré C, et al. Locally administered prostaglandin E2 prevents aeroallergen-induced airway sensitization in mice through immunomodulatory mechanisms. *Pharmacol Res.* (2013) 70:50–9. doi: 10.1016/j.phrs.2012.12.008
29. Kay LJ, Yeo WW, Peachell PT. Prostaglandin E 2activates EP 2receptors to inhibit human lung mast cell degranulation. *Br J Pharmacol.* (2006) 147:707–13. doi: 10.1038/sj.bjp.0706664
30. Sturm EM, Schratl P, Schuligoi R, Konya V, Sturm GJ, Lippe IT, et al. Prostaglandin E2 inhibits eosinophil trafficking through E-prostanoid 2 receptors. *J Immunol.* (2008) 181:7273–83. doi: 10.4049/jimmunol.181.10.7273
31. Nataraj C, Thomas DW, Tilley SL, Nguyen MT, Mannon R, Koller BH, et al. Receptors for prostaglandin E(2) that regulate cellular immune responses in the mouse. *J Clin Invest.* (2001) 108:1229–35. doi: 10.1172/JCI200113640
32. Zhou W, Blackwell TS, Goleniewska K, O'Neal JF, FitzGerald GA, Lucitt M, et al. Prostaglandin I 2analogs inhibit Th1 and Th2 effector cytokine production by CD4 T cells. *J Leukoc Biol.* (2006) 81:809–17. doi: 10.1189/jlb.0606375
33. Baratelli F, Lin Y, Zhu L, Yang SC, Heuze-Vourc'h N, Zeng G, et al. Prostaglandin E2 induces FOXP3 Gene expression and T regulatory cell function in human CD4+ T cells. *J Immunol.* (2005) 175:1483–90. doi: 10.4049/jimmunol.175.3.1483
34. Hammad H, Kool M, Soullié T, Narumiya S, Trottein F, Hoogsteden HC, et al. Activation of the D prostanoid 1 receptor suppresses asthma by modulation of lung dendritic cell function and induction of regulatory T cells. *J Exp Med.* (2007) 204:357–67. doi: 10.1084/jem.20061196
35. Yamabayashi C, Koya T, Kagamu H, Kawakami H, Kimura Y, Furukawa T, et al. A novel prostacyclin agonist protects against airway hyperresponsiveness and remodeling in mice. *Am J Respir Cell Mol Biol.* (2012) 47:170–7. doi: 10.1165/rcmb.2011-0350OC
36. Hattori R, Shimizu S, Majima Y, Shimizu T. Prostaglandin E2 receptor EP2, EP3, and EP4 agonists inhibit antigen-induced mucus hypersecretion in the nasal epithelium of sensitized rats. *Ann Otol Rhinol Laryngol.* (2009) 118:536–41. doi: 10.1177/000348940911800714
37. Lundequist A, Nallamshetty SN, Xing W, Feng C, Laidlaw TM, Uematsu S, et al. Prostaglandin E(2) exerts homeostatic regulation of pulmonary vascular remodeling in allergic airway inflammation. *J Immunol.* (2010) 184:433–41. doi: 10.4049/jimmunol.0902835
38. Huang SK, White ES, Wettlaufer SH, Grifka H, Hogaboam CM, Thannickal VJ, et al. Prostaglandin E 2induces fibroblast apoptosis by modulating multiple survival pathways. *FASEB J.* (2009) 23:4317–26. doi: 10.1096/fj.08-128801
39. Kolodsick JE, Peters-Golden M, Larios J, Toews GB, Thannickal VJ, Moore BB. Prostaglandin E 2inhibits fibroblast to myofibroblast transition via E. prostanoid receptor 2 signaling and cyclic adenosine monophosphate elevation. *Am J Respir Cell Mol Biol.* (2003) 29:537–44. doi: 10.1165/rcmb.2002-0243OC
40. Mori A, Ito S, Morioka M, Aso H, Kondo M, Sokabe M, et al. Effects of specific prostanoid EP receptor agonists on cell proliferation and intracellular Ca2+ concentrations in human airway smooth muscle cells. *Eur J Pharmacol.* (2011) 659:72–8. doi: 10.1016/j.ejphar.2011.03.001
41. Pavord ID, Wisniewski A, Mathur R, Wahedna I, Knox AJ, Tattersfield AE. Effect of inhaled prostaglandin E2 on bronchial reactivity to sodium metabisulphite and methacholine in patients with asthma. *Thorax* (1991) 46:633–7. doi: 10.1136/thx.46.9.633
42. Szczeklik A, Mastalerz L, Nizankowska E, Cmiel A. Protective and bronchodilator effects of prostaglandin E and salbutamol in aspirin-induced asthma. *Am J Respir Crit Care Med.* (1996) 153:567–71. doi: 10.1164/ajrccm.153.2.8564099
43. Sestini P, Armetti L, Gambaro G, Pieroni MG, Refini RM, Sala A, et al. Inhaled PGE2 prevents aspirin-induced bronchoconstriction and urinary LTE4 excretion in aspirin-sensitive asthma. *Am J Respir Crit Care Med.* (1996) 153:572–5. doi: 10.1164/ajrccm.153.2.8564100
44. Maher SA, Birrell MA, Belvisi MG. Prostaglandin E2 mediates cough via the EP3 receptor: implications for future disease therapy. *Am J Respir Crit Care Med.* (2009) 180:923–8. doi: 10.1164/rccm.200903-0388OC
45. Adelroth E, Morris MM, Hargreave FE, O'Byrne PM. Airway responsiveness to leukotrienes C4 and D4 and to methacholine in patients with asthma and normal controls. *N Engl J Med.* (1986) 315:480–4. doi: 10.1056/NEJM198608213150803
46. Hamilton A, Faiferman I, Stober P, Watson RM, O'Byrne PM. Pranlukast, a cysteinyl leukotriene receptor antagonist, attenuates allergen-induced early- and late-phase bronchoconstriction and airway hyperresponsiveness in asthmatic subjects. *J Allergy Clin Immunol.* (1998) 102:177–83. doi: 10.1016/S0091-6749(98)70083-1
47. Parameswaran K, Watson R, Gauvreau GM, Sehmi R, O'Byrne PM. The effect of pranlukast on allergen-induced bone marrow Eosinophilopoiesis in subjects with asthma. *Am J Respir Crit Care Med.* (2004) 169:915–20. doi: 10.1164/rccm.200312-1645OC

48. Leigh R, Vethanayagam D, Yoshida M, Watson RM, Rerecich T, Inman MD, et al. Effects of montelukast and budesonide on airway responses and airway inflammation in asthma. *Am J Respir Crit Care Med.* (2002) 166:1212–7. doi: 10.1164/rccm.200206-509OC
49. Gauvreau GM, Parameswaran KN, Watson RM, O'Byrne PM. Inhaled leukotriene E 4, but not leukotriene D 4, increased airway inflammatory cells in subjects with atopic asthma. *Am J Respir Crit Care Med.* (2001) 164:1495–500. doi: 10.1164/ajrccm.164.8.2102033
50. Wenzel SE, Larsen GL, Johnston K, Voelkel NF, Westcott JY. Elevated levels of leukotriene C 4in bronchoalveolar lavage fluid from atopic asthmatics after endobronchial allergen challenge. *Am Rev Respir Dis.* (1990) 142:112–9. doi: 10.1164/ajrccm/142.1.112
51. Manning PJ, Rokach J, Malo JL, Ethier D, Cartier A, Girard Y, et al. Urinary leukotriene E4 levels during early and late asthmatic responses. *J Allergy Clin Immunol.* (1990) 86:211–20. doi: 10.1016/S0091-6749(05)80068-5
52. Barnes N, Thomas M, Price D, Tate H. The national montelukast survey. *J Allergy Clin Immunol.* (2005) 115:47–54. doi: 10.1016/j.jaci.2004.10.011
53. Tager AM, Bromley SK, Medoff BD, Islam SA, Bercury SD, Friedrich EB, et al. Leukotriene B4 receptor BLT1 mediates early effector T cell recruitment. *Nat Immunol.* (2003) 4:982–90. doi: 10.1038/ni970
54. Goodarzi K, Goodarzi M, Tager AM, Luster AD, Andrian von UH. Leukotriene B4 and BLT1 control cytotoxic effector T cell recruitment to inflamed tissues. *Nat Immunol.* (2003) 4:965–973. doi: 10.1038/ni972
55. Miyahara N, Ohnishi H, Matsuda H, Miyahara S, Takeda K, Koya T, et al. Leukotriene B4 receptor 1 expression on dendritic cells is required for the development of Th2 responses and allergen-induced airway hyperresponsiveness. *J Immunol.* (2008) 181:1170–8. doi: 10.4049/jimmunol.181.2.1170
56. Terawaki K, Yokomizo T, Nagase T, Toda A, Taniguchi M, Hashizume K, et al. Absence of leukotriene B4 receptor 1 confers resistance to airway hyperresponsiveness and Th2-type immune responses. *J Immunol.* (2005) 175:4217–25. doi: 10.4049/jimmunol.175.7.4217
57. Planagumà A, Kazani S, Marigowda G, Haworth O, Mariani TJ, Israel E, et al. Airway lipoxin A4 generation and lipoxin A4 receptor expression are decreased in severe asthma. *Am J Respir Crit Care Med.* (2008) 178:574–82. doi: 10.1164/rccm.200801-061OC
58. Levy BD, Bonnans C, Silverman ES, Palmer LJ, Marigowda G, Israel E. Severe asthma research program, national heart, lung, and blood institute. Diminished lipoxin biosynthesis in severe asthma. *Am J Respir Crit Care Med.* (2005) 172:824–30. doi: 10.1164/rccm.200410-1413OC
59. Çelik GE, Erkekol FO, Mısırlıgil Z, Melli M. Lipoxin A4 levels in asthma: relation with disease severity and aspirin sensitivity. *Clin Exp Allergy* (2007) 37:1494–501. doi: 10.1111/j.1365-2222.2007.02806.x
60. Levy BD, De Sanctis GT, Devchand PR, Kim E, Ackerman K, Schmidt BA, et al. Multi-pronged inhibition of airway hyper-responsiveness and inflammation by lipoxin A(4). *Nat Med.* (2002) 8:1018–23. doi: 10.1038/nm748
61. Levy BD, Lukacs NW, Berlin AA, Schmidt B, Guilford WJ, Serhan CN, et al. Lipoxin A4 stable analogs reduce allergic airway responses viamechanisms distinct from CysLT1 receptor antagonism. *FASEB J.* (2007) 21:3877–84. doi: 10.1096/fj.07-8653com
62. Haworth O, Cernadas M, Yang R, Serhan CN, Levy BD. Resolvin E1 regulates interleukin 23, interferon-γ and lipoxin A4 to promote the resolution of allergic airway inflammation. *Nat Immunol.* (2008) 9:873–9. doi: 10.1038/ni.1627
63. Levy BD, Kohli P, Gotlinger K, Haworth O, Hong S, Kazani S, et al. Protectin D1 is generated in asthma and dampens airway inflammation and hyperresponsiveness. *J Immunol.* (2007) 178:496–502. doi: 10.4049/jimmunol.178.1.496
64. Rogerio AP, Haworth O, Croze R, Oh SF, Uddin M, Carlo T, et al. Resolvin D1 and aspirin-triggered resolvin D1 promote resolution of allergic airways responses. *J Immunol.* (2012) 189:1983–91. doi: 10.4049/jimmunol.1101665
65. Henderson WR, Chi EY, Bollinger JG, Tien Y-T, Ye X, Castelli L, et al. Importance of group X-secreted phospholipase A2 in allergen-induced airway inflammation and remodeling in a mouse asthma model. *J Exp Med.* (2007) 204:865–77. doi: 10.1084/jem.20070029
66. Muñoz NM, Meliton AY, Arm JP, Bonventre JV, Cho W, Leff AR. Deletion of secretory group V phospholipase A2 attenuates cell migration and airway hyperresponsiveness in immunosensitized mice. *J Immunol.* (2007) 179:4800–7. doi: 10.4049/jimmunol.179.7.4800
67. Hallstrand TS, Lai Y, Ni Z, Oslund RC, Henderson WR Jr, Gelb MH, et al. Relationship between levels of secreted phospholipase A2 groups IIA and X in the airways and asthma severity. *Clin Exp Allergy* (2011) 41:801–10. doi: 10.1111/j.1365-2222.2010.03676.x
68. Hallstrand TS, Chi EY, Singer AG, Gelb MH, Henderson WR. Secreted phospholipase A2 group X overexpression in asthma and bronchial hyperresponsiveness. *Am J Respir Crit Care Med.* (2007) 176:1072–8. doi: 10.1164/rccm.200707-1088OC
69. Giannattasio G, Fujioka D, Xing W, Katz HR, Boyce JA, Balestrieri B. Group V secretory phospholipase A2 reveals its role in house dust mite-induced allergic pulmonary inflammation by regulation of dendritic cell function. *J Immunol.* (2010) 185:4430–8. doi: 10.4049/jimmunol.1001384
70. Hammad H, Chieppa M, Perros F, Willart MA, Germain RN, Lambrecht BN. House dust mite allergen induces asthma via Toll-like receptor 4 triggering of airway structural cells. *Nat Med.* (2009) 15:410–6. doi: 10.1038/nm.1946
71. Lambrecht BN, Hammad H. Dendritic cell and epithelial cell interactions at the origin of murine asthma. *Ann Am Thorac Soc.* (2014) 11 (Suppl. 5):S236–43. doi: 10.1513/AnnalsATS.201405-218AW
72. Lambrecht BN, De Veerman M, Coyle AJ, Gutierrez-Ramos JC, Thielemans K, Pauwels RA. Myeloid dendritic cells induce Th2 responses to inhaled antigen, leading to eosinophilic airway inflammation. *J Clin Invest.* (2000) 106:551–9. doi: 10.1172/JCI8107
73. Raymond M, Van VQ, Wakahara K, Rubio M, Sarfati M. Lung dendritic cells induce T(H)17 cells that produce T(H)2 cytokines, express GATA-3, and promote airway inflammation. *J Allergy Clin Immunol.* (2011) 128:192–201.e6. doi: 10.1016/j.jaci.2011.04.029
74. Hammad H, Plantinga M, Deswarte K, Pouliot P, Willart MAM, Kool M, et al. Inflammatory dendritic cells–not basophils–are necessary and sufficient for induction of Th2 immunity to inhaled house dust mite allergen. *J Exp Med.* (2010) 207:2097–111. doi: 10.1084/jem.20101563
75. Plantinga M, Guilliams M, Vanheerswynghels M, Deswarte K, Branco-Madeira F, Toussaint W, et al. Conventional and monocyte-derived CD11b(+) dendritic cells initiate and maintain T helper 2 cell-mediated immunity to house dust mite allergen. *Immunity* (2013) 38:322–35. doi: 10.1016/j.immuni.2012.10.016
76. van Rijt LS, Vos N, Willart M, Muskens F, Tak PP, van der Horst C, et al. Persistent activation of dendritic cells after resolution of allergic airway inflammation breaks tolerance to inhaled allergens in mice. *Am J Respir Crit Care Med.* (2011) 184:303–11. doi: 10.1164/rccm.201101-0019OC
77. de Kleer IM, Kool M, de Bruijn MJW, Willart M, van Moorleghem J, Schuijs MJ, et al. Perinatal activation of the interleukin-33 pathway promotes type 2 immunity in the developing lung. *Immunity* (2016) 45:1285–98. doi: 10.1016/j.immuni.2016.10.031
78. Phythian-Adams AT, Cook PC, Lundie RJ, Jones LH, Smith KA, Barr TA, et al. CD11c depletion severely disrupts Th2 induction and development *in vivo*. *J Exp Med.* (2010) 207:2089–96. doi: 10.1084/jem.20100734
79. Tang H, Cao W, Kasturi SP, Ravindran R, Nakaya HI, Kundu K, et al. The T helper type 2 response to cysteine proteases requires dendritic cell-basophil cooperation via ROS-mediated signaling. *Nat Immunol.* (2010) 11:608–17. doi: 10.1038/ni.1883
80. van Rijt LS, Jung S, KleinJan A, Vos N, Willart M, Duez C, et al. *In vivo* depletion of lung CD11c+ dendritic cells during allergen challenge abrogates the characteristic features of asthma. *J Exp Med.* (2005) 201:981–91. doi: 10.1084/jem.20042311
81. Thornton EE, Looney MR, Bose O, Sen D, Sheppard D, Locksley R, et al. Spatiotemporally separated antigen uptake by alveolar dendritic cells and airway presentation to T cells in the lung. *J Exp Med.* (2012) 209:1183–99. doi: 10.1084/jem.20112667
82. Medoff BD, Seung E, Hong S, Thomas SY, Sandall BP, Duffield JS, et al. CD11b+ myeloid cells are the key mediators of Th2 cell homing into the airway in allergic inflammation. *J Immunol.* (2009) 182:623–35. doi: 10.4049/jimmunol.182.1.623
83. Guilliams M, Lambrecht BN, Hammad H. Division of labor between lung dendritic cells and macrophages in the defense against pulmonary infections. *Mucosal Immunol.* (2013) 6:464–73. doi: 10.1038/mi.2013.14

84. Williams JW, Tjota MY, Clay BS, Vander Lugt B, Bandukwala HS, Hrusch CL, et al. Transcription factor IRF4 drives dendritic cells to promote Th2 differentiation. *Nat Commun.* (2013) 4:2990. doi: 10.1038/ncomms3990
85. Schlitzer A, McGovern N, Teo P, Zelante T, Atarashi K, Low D, et al. IRF4 transcription factor-dependent CD11b+ dendritic cells in human and mouse control mucosal IL-17 cytokine responses. *Immunity* (2013) 38:970–83. doi: 10.1016/j.immuni.2013.04.011
86. Norimoto A, Hirose K, Iwata A, Tamachi T, Yokota M, Takahashi K, et al. Dectin-2 promotes house dust mite-induced T helper type 2 and type 17 cell differentiation and allergic airway inflammation in mice. *Am J Respir Cell Mol Biol.* (2014) 51:201–9. doi: 10.1165/rcmb.2013-0522OC
87. Nakano H, Burgents JE, Nakano K, Whitehead GS, Cheong C, Bortner CD, et al. Migratory properties of pulmonary dendritic cells are determined by their developmental lineage. *Mucosal Immunol.* (2013) 6:678–91. doi: 10.1038/mi.2012.106
88. Everts B, Tussiwand R, Dreesen L, Fairfax KC, Huang SC-C, Smith AM, et al. Migratory CD103+ dendritic cells suppress helminth-driven type 2 immunity through constitutive expression of IL-12. *J Exp Med.* (2016) 213:35–51. doi: 10.1084/jem.20150235
89. Semmrich M, Plantinga M, Svensson-Frej M, Uronen-Hansson H, Gustafsson T, Mowat AM, et al. Directed antigen targeting *in vivo* identifies a role for CD103+ dendritic cells in both tolerogenic and immunogenic T-cell responses. *Mucosal Immunol.* (2012) 5:150–60. doi: 10.1038/mi.2011.61
90. Khare A, Krishnamoorthy N, Oriss TB, Fei M, Ray P, Ray A. Cutting edge: inhaled antigen upregulates retinaldehyde dehydrogenase in lung CD103+ but not plasmacytoid dendritic cells to induce Foxp3 *de novo* in CD4+ T cells and promote airway tolerance. *J Immunol.* (2013) 191:25–9. doi: 10.4049/jimmunol.1300193
91. de Heer HJ, Hammad H, Soullié T, Hijdra D, Vos N, Willart MAM, et al. Essential role of lung plasmacytoid dendritic cells in preventing asthmatic reactions to harmless inhaled antigen. *J Exp Med.* (2004) 200:89–98. doi: 10.1084/jem.20040035
92. Oriss TB, Ostroukhova M, Seguin-Devaux C, Dixon-McCarthy B, Stolz DB, Watkins SC, et al. Dynamics of dendritic cell phenotype and interactions with CD4+ T cells in airway inflammation and tolerance. *J Immunol.* (2005) 174:854–63. doi: 10.4049/jimmunol.174.2.854
93. Kool M, van Nimwegen M, Willart MAM, Muskens F, Boon L, Smit JJ, et al. An anti-inflammatory role for plasmacytoid dendritic cells in allergic airway inflammation. *J Immunol.* (2009) 183:1074–82. doi: 10.4049/jimmunol.0900471
94. Lombardi V, Speak AO, Kerzerho J, Szely N, Akbari O. $CD8\alpha^{+}\beta^{-}$ and $CD8\alpha^{+}\beta^{+}$ plasmacytoid dendritic cells induce $Foxp3^{+}$ regulatory T cells and prevent the induction of airway hyper-reactivity. *Mucosal Immunol.* (2012) 5:432–43. doi: 10.1038/mi.2012.20
95. Lambrecht BN, Hammad H. The airway epithelium in asthma. *Nat Med.* (2012) 18:684–92. doi: 10.1038/nm.2737
96. Hammad H, Lambrecht BN. Barrier epithelial cells and the control of type 2 immunity. *Immunity* (2015) 43:29–40. doi: 10.1016/j.immuni.2015.07.007
97. Willart MAM, Deswarte K, Pouliot P, Braun H, Beyaert R, Lambrecht BN, et al. Interleukin-1α controls allergic sensitization to inhaled house dust mite via the epithelial release of GM-CSF and IL-33. *J Exp Med.* (2012) 209:1505–17. doi: 10.1084/jem.20112691
98. Schuijs MJ, Willart MA, Vergote K, Gras D, Deswarte K, Ege MJ, et al. Farm dust and endotoxin protect against allergy through A20 induction in lung epithelial cells. *Science* (2015) 349:1106–10. doi: 10.1126/science.aac6623
99. Scandella E, Men Y, Legler DF, Gillessen S, Prikler L, Ludewig B, et al. CCL19/CCL21-triggered signal transduction and migration of dendritic cells requires prostaglandin E2. *Blood* (2004) 103:1595–601. doi: 10.1182/blood-2003-05-1643
100. van Helden SFG, Krooshoop DJEB, Broers KCM, Raymakers RAP, Figdor CG, van Leeuwen FN. A critical role for prostaglandin E2 in podosome dissolution and induction of high-speed migration during dendritic cell maturation. *J Immunol.* (2006) 177:1567–74. doi: 10.4049/jimmunol.177.3.1567
101. Legler DF, Krause P, Scandella E, Singer E, Groettrup M. Prostaglandin E2 is generally required for human dendritic cell migration and exerts its effect via EP2 and EP4 receptors. *J Immunol.* (2006) 176:966–73. doi: 10.4049/jimmunol.176.2.966
102. Harizi H, Grosset C, Gualde N. Prostaglandin E2 modulates dendritic cell function via EP2 and EP4 receptor subtypes. *J Leukoc Biol.* (2003) 73:756–63. doi: 10.1189/jlb.1002483
103. Angeli V, Faveeuw C, Roye O, Fontaine J, Teissier E, Capron A, et al. Role of the parasite-derived prostaglandin D2 in the inhibition of epidermal Langerhans cell migration during schistosomiasis infection. *J Exp Med.* (2001) 193:1135–47. doi: 10.1084/jem.193.10.1135
104. Hammad H, de Heer HJ, Soullié T, Hoogsteden HC, Trottein F, Lambrecht BN. Prostaglandin D2 inhibits airway dendritic cell migration and function in steady state conditions by selective activation of the D prostanoid receptor 1. *J Immunol.* (2003) 171:3936–40. doi: 10.4049/jimmunol.171.8.3936
105. Robbiani DF, Finch RA, Jäger D, Muller WA, Sartorelli AC, Randolph GJ. The leukotriene C4 transporter MRP1 regulates CCL19 (MIP-3β, ELC)–dependent mobilization of dendritic cells to lymph nodes. *Cell* (2000) 103:757–68. doi: 10.1016/S0092-8674(00)00179-3
106. Aliberti J, Hieny S, Reis e Sousa C, Serhan CN, Sher A. Lipoxin-mediated inhibition of IL-12 production by DCs: a mechanism for regulation of microbial immunity. *Nat Immunol.* (2002) 3:76–82. doi: 10.1038/ni745
107. Hilkens C, Snijders A, Vermeulen H, Meide PVD, Wierenga E, Kapsenberg M. Accessory cell-derived interleukin-12 and prostaglandin E 2determine the level of interferon- ? produced by activated human CD4 +T cells. *Ann N Y Acad Sci.* (1996) 795:349–50. doi: 10.1111/j.1749-6632.1996.tb52689.x
108. van der Pouw Kraan TC. Prostaglandin-E2 is a potent inhibitor of human interleukin 12 production. *J Exp Med.* (1995) 181:775–9. doi: 10.1084/jem.181.2.775
109. Kalinski P, Hilkens CM, Snijders A, Snijdewint FG, Kapsenberg ML. Dendritic cells, obtained from peripheral blood precursors in the presence of PGE2, promote Th2 responses. *Adv Exp Med Biol.* (1997) 417:363–7.
110. Kalinski P, Hilkens CM, Snijders A, Snijdewint FG, Kapsenberg ML. IL-12-deficient dendritic cells, generated in the presence of prostaglandin E2, promote type 2 cytokine production in maturing human naive T helper cells. *J Immunol.* (1997) 159:28–35.
111. Kalinski P, Schuitemaker JH, Hilkens CM, Kapsenberg ML. Prostaglandin E2 induces the final maturation of IL-12-deficient CD1a+CD83+ dendritic cells: the levels of IL-12 are determined during the final dendritic cell maturation and are resistant to further modulation. *J Immunol.* (1998) 161:2804–9.
112. Vieira PL, de Jong EC, Wierenga EA, Kapsenberg ML, Kalinski P. Development of Th1-inducing capacity in myeloid dendritic cells requires environmental instruction. *J Immunol.* (2000) 164:4507–12. doi: 10.4049/jimmunol.164.9.4507
113. Hedi H, Norbert G. Inhibition of IL-6, TNF-α, and cyclooxygenase-2 protein expression by prostaglandin E2-induced IL-10 in bone marrow-derived dendritic cells. *Cell Immunol.* (2004) 228:99–109. doi: 10.1016/j.cellimm.2004.04.003
114. Vassiliou E, Jing H, Ganea D. Prostaglandin E2 inhibits TNF production in murine bone marrow-derived dendritic cells. *Cell Immunol.* (2003) 223:120–32. doi: 10.1016/S0008-8749(03)00158-8
115. Harizi H, Juzan M, Pitard V, Moreau J-F, Gualde N. Cyclooxygenase-2-issued prostaglandin e(2) enhances the production of endogenous IL-10, which down-regulates dendritic cell functions. *J Immunol.* (2002) 168:2255–63. doi: 10.4049/jimmunol.168.5.2255
116. Walker W, Rotondo D. Prostaglandin E2 is a potent regulator of interleukin-12- and interleukin-18-induced natural killer cell interferon-gamma synthesis. *Immunology* (2004) 111:298–305. doi: 10.1111/j.1365-2567.2004.01810.x
117. Rieser C, Papesh C, Herold M, Böck G, Ramoner R, Klocker H, et al. Differential deactivation of human dendritic cells by endotoxin desensitization: role of tumor necrosis factor-alpha and prostaglandin E2. *Blood* (1998) 91:3112–7.
118. Rieser C, Böck G, Klocker H, Bartsch G, Thurnher M. Prostaglandin E2 and tumor necrosis factor alpha cooperate to activate human dendritic cells: synergistic activation of interleukin 12 production. *J Exp Med.* (1997) 186:1603–8.
119. Steinbrink K, Paragnik L, Jonuleit H, Tüting T, Knop J, Enk AH. Induction of dendritic cell maturation and modulation of dendritic cell-induced

immune responses by prostaglandins. *Arch Dermatol Res.* (2000) 292:437–45. doi: 10.1007/s004030002920437.403
120. Lee YG, Jeong JJ, Nyenhuis S, Berdyshev E, Chung S, Ranjan R, et al. Recruited alveolar macrophages, in response to airway epithelial–derived monocyte chemoattractant protein 1/CCL2, regulate airway inflammation and remodeling in allergic asthma. *Am J Respir Cell Mol Biol.* (2015) 52:772–84. doi: 10.1165/rcmb.2014-0255OC
121. Rubio MT, Means TK, Chakraverty R, Shaffer J, Fudaba Y, Chittenden M, et al. Maturation of human monocyte-derived dendritic cells (MoDCs) in the presence of prostaglandin E2 optimizes CD4 and CD8 T cell-mediated responses to protein antigens: role of PGE2 in chemokine and cytokine expression by MoDCs. *Int Immunol.* (2005) 17:1561–72. doi: 10.1093/intimm/dxh335
122. Khayrullina T, Yen J-H, Jing H, Ganea D. *In vitro* differentiation of dendritic cells in the presence of prostaglandin E2 alters the IL-12/IL-23 balance and promotes differentiation of Th17 cells. *J Immunol.* (2008) 181:721–35. doi: 10.4049/jimmunol.181.1.721
123. Sheibanie AF, Tadmori I, Jing H, Vassiliou E, Ganea D. Prostaglandin E2 induces IL-23 production in bone marrow-derived dendritic cells. *FASEB J.* (2004) 18:1318–20. doi: 10.1096/fj.03-1367fje
124. Betz M, Fox BS. Prostaglandin E2 inhibits production of Th1 lymphokines but not of Th2 lymphokines. *J Immunol.* (1991) 146:108–13.
125. Snijdewint FG, Kalinski P, Wierenga EA, Bos JD, Kapsenberg ML. Prostaglandin E2 differentially modulates cytokine secretion profiles of human T helper lymphocytes. *J Immunol.* (1993) 150:5321–9.
126. Zasłona Z, Okunishi K, Bourdonnay E, Domingo-Gonzalez R, Moore BB, Lukacs NW, et al. Prostaglandin E2 suppresses allergic sensitization and lung inflammation by targeting the E prostanoid 2 receptor on T cells. *J Allergy Clin Immunol.* (2014) 133:379–87. doi: 10.1016/j.jaci.2013.07.037
127. Boniface K, Bak-Jensen KS, Li Y, Blumenschein WM, McGeachy MJ, McClanahan TK, et al. Prostaglandin E2 regulates Th17 cell differentiation and function through cyclic AMP and EP2/EP4 receptor signaling. *J Exp Med.* (2009) 206:535–48. doi: 10.1084/jem.20082293
128. Tanaka K, Hirai H, Takano S, Nakamura M, Nagata K. Effects of prostaglandin D2 on helper T cell functions. *Biochem Biophy Res Commun.* (2004) 316:1009–14. doi: 10.1016/j.bbrc.2004.02.151
129. Jaffar Z, Wan K-S, Roberts K. A key role for prostaglandin I2 in limiting lung mucosal Th2, but not Th1, responses to inhaled allergen. *J Immunol.* (2002) 169:5997–6004. doi: 10.4049/jimmunol.169.10.5997
130. Machida I, Matsuse H, Kondo Y, Kawano T, Saeki S, Tomari S, et al. Cysteinyl leukotrienes regulate dendritic cell functions in a murine model of asthma. *J Immunol.* (2004) 172:1833–8. doi: 10.4049/jimmunol.172.3.1833
131. Okunishi K, Dohi M, Nakagome K, Tanaka R, Yamamoto K. A novel role of cysteinyl leukotrienes to promote dendritic cell activation in the antigen-induced immune responses in the lung. *J Immunol.* (2004) 173:6393–402. doi: 10.4049/jimmunol.173.10.6393
132. Barrett NA, Rahman OM, Fernandez JM, Parsons MW, Xing W, Austen KF, et al. Dectin-2 mediates Th2 immunity through the generation of cysteinyl leukotrienes. *J Exp Med.* (2011) 208:593–604. doi: 10.1084/jem.20100793
133. Doherty TA, Khorram N, Lund S, Mehta AK, Croft M, Broide DH. Lung type 2 innate lymphoid cells express cysteinyl leukotriene receptor 1, which regulates TH2 cytokine production. *J Allergy Clin Immunol.* (2013) 132:205–13. doi: 10.1016/j.jaci.2013.03.048
134. Prinz I, Gregoire C, Mollenkopf H, Aguado E, Wang Y, Malissen M, et al. The type 1 cysteinyl leukotriene receptor triggers calcium influx and chemotaxis in mouse alpha beta- and gamma delta effector T cells. *J Immunol.* (2005) 175:713–9. doi: 10.4049/jimmunol.175.2.713
135. Yamaoka KA, Kolb J-P. Leukotriene B4 induces interleukin 5 generation from human T lymphocytes. *Eur J Immunol.* (1993) 23:2392–8. doi: 10.1002/eji.1830231003
136. Barrett NA, Fernandez JM, Maekawa A, Xing W, Li L, Parsons MW, et al. Cysteinyl leukotriene 2 receptor on dendritic cells negatively regulates ligand-dependent allergic pulmonary inflammation. *J Immunol.* (2012) 189:4556–65. doi: 10.4049/jimmunol.1201865
137. Cai Y, Kumar RK, Zhou J, Foster PS, Webb DC. Ym1/2 promotes Th2 cytokine expression by inhibiting 12/15(S)-lipoxygenase: identification of a novel pathway for regulating allergic inflammation. *J Immunol.* (2009) 182:5393–9. doi: 10.4049/jimmunol.0803874
138. Linton P-J, Bautista B, Biederman E, Bradley ES, Harbertson J, Kondrack RM, et al. Costimulation via OX40L expressed by B cells is sufficient to determine the extent of primary CD4 cell expansion and Th2 cytokine secretion *in vivo*. *J Exp Med.* (2003) 197:875–83. doi: 10.1084/jem.20021290
139. Rodríguez-Pinto D, Moreno J. B cells can prime naive CD4 +T cells *in vivo* in the absence of other professional antigen-presenting cells in a CD154-CD40-dependent manner. *Eur J Immunol.* (2005) 35:1097–105. doi: 10.1002/eji.200425732
140. Liu Q, Liu Z, Rozo CT, Hamed HA, Alem F, Urban JF, et al. The role of B cells in the development of CD4 effector T cells during a polarized Th2 immune response. *J Immunol.* (2007) 179:3821–30. doi: 10.4049/jimmunol.179.6.3821
141. Dwyer DF, Woodruff MC, Carroll MC, Austen KF, Gurish MF. B cells regulate CD4+ T cell responses to papain following B cell receptor-independent papain uptake. *J Immunol.* (2014) 193:529–39. doi: 10.4049/jimmunol.1303247
142. Dullaers M, Schuijs MJ, Willart M, Fierens K, van Moorleghem J, Hammad H, et al. House dust mite-driven asthma and allergen-specific T cells depend on B cells when the amount of inhaled allergen is limiting. *J Allergy Clin Immunol.* (2017) 140:76–88.e7. doi: 10.1016/j.jaci.2016.09.020
143. Yang Q, Shi M, Shen Y, Cao Y, Zuo S, Zuo C, et al. COX-1-derived thromboxane A2 plays an essential role in early B-cell development via regulation of JAK/STAT5 signaling in mouse. *Blood* (2014) 124:1610–21. doi: 10.1182/blood-2014-03-559658
144. Blaho VA, Buczynski MW, Dennis EA, Brown CR. Cyclooxygenase-1 orchestrates germinal center formation and antibody class-switch via regulation of IL-17. *J Immunol.* (2009) 183:5644–53. doi: 10.4049/jimmunol.0901499
145. Fedyk ER, Phipps RP. Prostaglandin E2 receptors of the EP2 and EP4 subtypes regulate activation and differentiation of mouse B lymphocytes to IgE-secreting cells. *Proc Natl Acad Sci USA.* (1996) 93:10978–83. doi: 10.1073/pnas.93.20.10978
146. Gao Y, Nish SA, Jiang R, Hou L, Licona-Limón P, Weinstein JS, et al. Control of T helper 2 responses by transcription factor IRF4-dependent dendritic cells. *Immunity* (2013) 39:722–32. doi: 10.1016/j.immuni.2013.08.028
147. Hajek AR, Lindley AR, Favoreto S, Carter R, Schleimer RP, Kuperman DA. 12/15-Lipoxygenase deficiency protects mice from allergic airways inflammation and increases secretory IgA levels. *J Allergy Clin Immunol.* (2008) 122:633–9.e3. doi: 10.1016/j.jaci.2008.06.021
148. Roper RL, Phipps RP. Prostaglandin E2 and cAMP inhibit B lymphocyte activation and simultaneously promote IgE and IgG1 synthesis. *J Immunol.* (1992) 149:2984–91.
149. Roper RL, Brown DM, Phipps RP. Prostaglandin E2 promotes B lymphocyte Ig isotype switching to IgE. *J Immunol.* (1995) 154:162–70.
150. Roper RL, Conrad DH, Brown DM, Warner GL, Phipps RP. Prostaglandin E2 promotes IL-4-induced IgE and IgG1 synthesis. *J Immunol.* (1990) 145:2644–51.
151. Gao Y, Zhao C, Wang W, Jin R, Li Q, Ge Q, et al. Prostaglandins E2 signal mediated by receptor subtype EP2 promotes IgE production *in vivo* and contributes to asthma development. *Sci Rep.* (2016) 6:20505. doi: 10.1038/srep20505
152. Murn J, Alibert O, Wu N, Tendil S, Gidrol X. Prostaglandin E2 regulates B cell proliferation through a candidate tumor suppressor, Ptger4. *J Exp Med.* (2008) 205:3091–103. doi: 10.1084/jem.20081163
153. Lamoureux J, Stankova J, Rolapleszczynski M. Leukotriene D4 enhances immunoglobulin production in CD40-activated human B lymphocytes. *J Allergy Clin Immunol.* (2006) 117:924–30. doi: 10.1016/j.jaci.2005.12.1329
154. Mirchandani AS, Besnard A-G, Yip E, Scott C, Bain CC, Cerovic V, et al. Type 2 innate lymphoid cells drive CD4+ Th2 cell responses. *J Immunol.* (2014) 192:2442–8. doi: 10.4049/jimmunol.1300974
155. Oliphant CJ, Hwang YY, Walker JA, Salimi M, Wong SH, Brewer JM, et al. MHCII-mediated dialog between group 2 innate lymphoid cells and CD4(+) T cells potentiates type 2 immunity and promotes parasitic helminth expulsion. *Immunity* (2014) 41:283–95. doi: 10.1016/j.immuni.2014.06.016
156. Barnig C, Cernadas M, Dutile S, Liu X, Perrella MA, Kazani S, et al. Lipoxin A4 regulates natural killer cell and type 2 innate lymphoid cell activation in asthma. *Sci Transl Med.* (2013) 5:174ra26. doi: 10.1126/scitranslmed.3004812
157. Salimi M, Stöger L, Liu W, Go S, Pavord I, Klenerman P, et al. Cysteinyl leukotriene E4 activates human group 2 innate lymphoid cells and enhances

the effect of prostaglandin D2 and epithelial cytokines. *J Allergy Clin Immunol.* (2017) 140:1090–100.e11. doi: 10.1016/j.jaci.2016.12.958
158. Maric J, Ravindran A, Mazzurana L, Björklund ÅK, Van Acker A, Rao A, et al. Prostaglandin E 2 suppresses human group 2 innate lymphoid cell function. *J Allergy Clin Immunol.* (2017) 141:1761–73.e6. doi: 10.1016/j.jaci.2017.09.050
159. Zhou W, Toki S, Zhang J, Goleniewksa K, Newcomb DC, Cephus JY, et al. Prostaglandin I2 signaling and inhibition of group 2 innate lymphoid cell responses. *Am J Respir Crit Care Med.* (2016) 193:31–42. doi: 10.1164/rccm.201410-1793OC
160. Lukic A, Larssen P, Fauland A, Samuelsson B, Wheelock CE, Gabrielsson S, et al. GM-CSF- and M-CSF-primed macrophages present similar resolving but distinct inflammatory lipid mediator signatures. *FASEB J.* (2017) 31:4370–81. doi: 10.1096/fj.201700319R
161. Guilliams M, De Kleer I, Henri S, Post S, Vanhoutte L, De Prijck S, et al. Alveolar macrophages develop from fetal monocytes that differentiate into long-lived cells in the first week of life via GM-CSF. *J Exp Med.* (2013) 210:1977–92. doi: 10.1084/jem.20131199
162. van de Laar L, Saelens W, De Prijck S, Martens L, Scott CL, Van Isterdael G, et al. Yolk sac macrophages, fetal liver, and adult monocytes can colonize an empty niche and develop into functional tissue-resident macrophages. *Immunity* (2016) 44:755–68. doi: 10.1016/j.immuni.2016.02.017
163. Westphalen K, Gusarova GA, Islam MN, Subramanian M, Cohen TS, Prince AS, et al. Sessile alveolar macrophages communicate with alveolar epithelium to modulate immunity. *Nature* (2014) 506:503–6. doi: 10.1038/nature12902
164. Bhattacharya J, Westphalen K. Macrophage-epithelial interactions in pulmonary alveoli. *Semin Immunopathol.* (2016) 38:461–9. doi: 10.1007/s00281-016-0569-x
165. Upham JW, Strickland DH, Bilyk N, Robinson BW, Holt PG. Alveolar macrophages from humans and rodents selectively inhibit T-cell proliferation but permit T-cell activation and cytokine secretion. *Immunology* (1995) 84:142–7.
166. Tang C, Ward C, Reid D, Bish R, O'Byrne PM, Walters EH. Normally suppressing CD40 coregulatory signals delivered by airway macrophages to TH2 lymphocytes are defective in patients with atopic asthma. *J Allergy Clin Immunol.* (2001) 107:863–70. doi: 10.1067/mai.2001.114987
167. Tang C, Inman MD, van Rooijen N, Yang P, Shen H, Matsumoto K, et al. Th type 1-stimulating activity of lung macrophages inhibits Th2-mediated allergic airway inflammation by an IFN-gamma-dependent mechanism. *J Immunol.* (2001) 166:1471–81. doi: 10.4049/jimmunol.166.3.1471
168. Zasłona Z, Przybranowski S, Wilke C, Van Rooijen N, Teitz-Tennenbaum S, Osterholzer JJ, et al. Resident alveolar macrophages suppress, whereas recruited monocytes promote, allergic lung inflammation in murine models of asthma. *J Immunol.* (2014) 193:4245–53. doi: 10.4049/jimmunol.1400580
169. Bourdonnay E, Zasłona Z, Penke LRK, Speth JM, Schneider DJ, Przybranowski S, et al. Transcellular delivery of vesicular SOCS proteins from macrophages to epithelial cells blunts inflammatory signaling. *J Exp Med.* (2015) 212:729–42. doi: 10.1084/jem.20141675
170. Speth JM, Bourdonnay E, Penke LRK, Mancuso P, Moore BB, Weinberg JB, et al. Alveolar epithelial cell-derived prostaglandin E2 serves as a request signal for macrophage secretion of suppressor of cytokine signaling 3 during innate inflammation. *J Immunol.* (2016) 196:5112–20. doi: 10.4049/jimmunol.1502153
171. Draijer C, Boorsma CE, Reker-Smit C, Post E, Poelstra K, Melgert BN. PGE2-treated macrophages inhibit development of allergic lung inflammation in mice. *J Leukoc Biol.* (2016) 100:95–102. doi: 10.1189/jlb.3MAB 1115-505R
172. Ohta S, Imamura M, Xing W, Boyce JA, Balestrieri B. Group V secretory phospholipase A2 is involved in macrophage activation and is sufficient for macrophage effector functions in allergic pulmonary inflammation. *J Immunol.* (2013) 190:5927–38. doi: 10.4049/jimmunol.1203202
173. Yamaguchi M, Zacharia J, Laidlaw TM, Balestrieri B. PLA2G5 regulates transglutaminase activity of human IL-4-activated M2 macrophages through PGE2 generation. *J Leukoc Biol.* (2016) 100:131–41. doi: 10.1189/jlb.3A0815-372R
174. Lauzon-Joset J-F, Marsolais D, Langlois A, Bissonnette EY. Dysregulation of alveolar macrophages unleashes dendritic cell-mediated mechanisms of allergic airway inflammation. *Mucosal Immunol.* (2014) 7:155–64. doi: 10.1038/mi.2013.34
175. Samuchiwal SK, Balestrieri B, Raff H, Boyce JA. Endogenous prostaglandin E 2amplifies IL-33 production by macrophages through an E prostanoid (EP) 2/EP 4-cAMP-EPAC-dependent pathway. *J Biol Chem.* (2017) 292:8195–206. doi: 10.1074/jbc.M116.769422
176. Jandl K, Stacher E, Bálint Z, Sturm EM, Maric J, Peinhaupt M, et al. Activated prostaglandin D2 receptors on macrophages enhance neutrophil recruitment into the lung. *J Allergy Clin Immunol.* (2016) 137:833–43. doi: 10.1016/j.jaci.2015.11.012
177. Johnston SL, Pattemore PK, Sanderson G, Smith S, Lampe F, Josephs L, et al. Community study of role of viral infections in exacerbations of asthma in 9-11 year old children. *BMJ* (1995) 310:1225–9. doi: 10.1136/bmj.310.6989.1225
178. Shiraishi Y, Asano K, Niimi K, Fukunaga K, Wakaki M, Kagyo J, et al. Cyclooxygenase-2/prostaglandin D_2/CRTH2 pathway mediates double-stranded RNA-induced enhancement of allergic airway inflammation. *J Immunol.* (2008) 180:541–9. doi: 10.4049/jimmunol.180.1.541
179. Shirey KA, Pletneva LM, Puche AC, Keegan AD, Prince GA, Blanco JCG, et al. Control of RSV-induced lung injury by alternatively activated macrophages is IL-4Rα-, TLR4- and IFN-β-dependent. *Mucosal Immunol.* (2010) 3:291–300. doi: 10.1038/mi.2010.6
180. Shirey KA, Lai W, Pletneva LM, Karp CL, Divanovic S, Blanco JCG, et al. Role of the lipoxygenase pathway in RSV-induced alternatively activated macrophages leading to resolution of lung pathology. *Mucosal Immunol.* (2013) 7:549–57. doi: 10.1038/mi.2013.71
181. Clarke DL, Davis NHE, Campion CL, Foster ML, Heasman SC, Lewis AR, et al. Dectin-2 sensing of house dust mite is critical for the initiation of airway inflammation. *Mucosal Immunol.* (2014) 7:558–67. doi: 10.1038/mi.2013.74

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 11 December 2018
doi: 10.3389/fimmu.2018.02816

Pollen Lipids Can Play a Role in Allergic Airway Inflammation

*Åslög Dahl**

Department of Biological and Environmental Sciences, University of Gothenburg, Gothenburg, Sweden

In seed plants, pollen grains carry the male gametes to female structures. They are frequent in the ambient air, and cause airway inflammation in one out of four persons in the population. This was traditionally attributed to soluble glycoproteins, leaking into the nasal mucosa or the conjunctiva, and able to bind antibodies. It is now more and more recognized that also other immunomodulating compounds are present. Lipids bind to Toll-like and PPARγ receptors belonging to antigen-presenting cells in the mammal immune system, activate invariant Natural Killer T-cells, and are able to induce a Type 2 reaction in effector cells. They may also mimic lipid mediators from mammal mast cells. Pollen grains have a rich lipodome of their own. Among the lipids that have been associated with an atopic reaction are saturated and unsaturated fatty acids, glycophospholipids, sphingolipids, sterols, and oxylipids, as well as lipopolysaccharides from the microbiome on the pollen surface. Lipids can be ligands to allergenic proteins.

Keywords: pollen, allergy, immunomodulatory lipids, lipid-binding allergens, pollen-associated lipid mediators, C1d receptros, iNKT-cells

OPEN ACCESS

Edited by:
Nestor González Roldán, Forschungszentrum Borstel, Germany

Reviewed by:
Paula Branquinho Andrade, Universidade do Porto, Portugal
Carmen Galan, Universidad de Córdoba, Spain

***Correspondence:**
Åslög Dahl
aslog.dahl@bioenv.gu.se

Specialty section:
This article was submitted to Inflammation, a section of the journal Frontiers in Immunology

Received: *22 August 2018*
Accepted: *14 November 2018*
Published: *11 December 2018*

Citation:
Dahl Å (2018) Pollen Lipids Can Play a Role in Allergic Airway Inflammation. Front. Immunol. 9:2816. doi: 10.3389/fimmu.2018.02816

About 20–25% of the population suffers from pollen allergy (1), with subsequent reduction of their quality of life and with large societal costs. The risk for sensitization to pollen depends to the degree of exposure. Thus, the main culprits are pollen borne in the air.

The traditional focus has been glycoproteins that are able to bind specific antibodies, quick to be eluted in moisture, before the pollen is eliminated by tears, mucus, or a sneeze. They have been localized to the cytoplasm and to the pollen wall. It is now recognized that also other immunomodulatory compounds emanate from the pollen grains. Among these are lipids, also able to interact with components of the innate immune system. The pollen grain has a lipidome of its own. In this review the main pollen lipids, their location in the pollen grain, and their biological functions are in focus, as well as observed effects on mammal immune reactions.

Lipids are non-polar molecules, which means their ends are not charged, and thus they will be soluble in non-polar solvents rather than in water, which in contrast is polar (2).

WHAT IS A POLLEN GRAIN?

A pollen grain carries the male gametes to the female reproductive structures. Seed plants have an internal fertilization that evolved in parallel to that found in animals during adaption to terrestrial life. During sexual reproduction, an immotile sperm cell, or in a number of gymnosperms, a spermatozoid, unites with an egg cell inside the tissues of a parental plant, independently of the presence of free-standing water. For this to happen, the pollen grain produces a pollen tube, which acts as a conduit for sperm cells to the egg cell. In seed plants, egg cells are contained within ovules. In gymnosperms, the ovules are born solitary or on the surface of scales often positioned in cones.

The pollen grain is delivered at the micropyle, a small opening in the surface of an ovule, which then is penetrated by the pollen tube. In angiosperms, ovules are enclosed in the ovary of the pistil, and therefore, the distance the pollen tube must grow is longer. The pollen grains are generally captured on the stigmatic surface of the pistil, where pollen tubes penetrate into its style.

In contrast to the situation in mammals, the haploid daughter cells from a meiotic division in plants are spores, which divide mitotically as to produce a multicellular, haploid individual that in due course will produce gametes, i.e., is a gametophyte. After gametic fusion, the diploid state is restored. The resulting zygote will divide mitotically and give rise to a multicellular plant, the sporophyte, forming sporangia where the aforementioned meiosis takes place. This alternation between a multicellular diploid and a multicellular haploid phase is called an alternation of generations. In seed plants, gametophytes are dependent on the sporophyte, unisexual, and with few cells. Their entire development takes place within the sporangium.

A pollen grain is a male gametophyte, with an outer wall and coating formed by sporophytic cells. In gymnosperms, microsporangia are usually situated in cone-like structures, whereas in angiosperms, they are identical to the four pollen sacs of the anther. The inside of the sporangium is lined with cells, which provide the microspores with nutrients, and later with wall and coat material. In angiosperms, the first mitosis of the microspore results in a small generative cell, and a much larger vegetative cell, which increases further in size due to an enlargement of the cytoplasm. The generative cell divides mitotically into two sperm cells with large nuclei and comparatively little cytoplasm. This division takes place before or after anther dehiscence and pollen germination, according to species. In gymnosperms, the number of mitotic divisions that produce the male gametophyte typically are two to five. In Taxales pollen (e.g., *Cupressus*, *Juniperus*), there is no vegetative cell. The generative cell in gymnosperms divides after pollen sac dehiscence and generates two sperm cells or spermatozoids.

THE MATURE POLLEN GRAIN AND ITS LIPIDOME

The Vegetative Cell of the Gametophyte

The vegetative cell of angiosperm pollen is the largest one, with a distal plasmalemma, a large central nucleus and a cytoplasm packed with mitochondria, plastids and storage organelles, and surrounding the two sperm cells (**Figure 1**). The sperms cells have plasmalemmae of their own, and are further enclosed in a plasmalemma from the vegetative cell.

The endoplasmatic reticulum (ER) of the vegetative cell is involved in the synthesis and transport of proteins and lipids. It is extensive, and extends from the plasma membrane surrounding the cell, through the cytoplasm, and forms a continuous connection with the nuclear envelope. The basic units of most of the complex structural lipids of the plasma membrane and the typically large endoplasmatic reticulum are fatty acids, synthesized *de novo* in the chloroplast stroma. In the mature pollen grain, the dominating fatty acids of these structures are octadecadienoic (linoleic; 18:2, *cis*-9,12) and hexadecanoic (palmitic; 16:0) acids (3).

Of the complex structural lipids in membranes, the glycerophospholipids are the most prominent. Their fatty acids are commonly polyunsaturated of C16/C18 type. Glycerophospholipids also usually have an additional group esterified to the phosphate, constituting a polar head group on the lipid molecule. Commonly found head groups in lipids of the pollen membrane are choline, etanolamine, serine, glycerol, and inositol. Because of their bipolarity, the phospholipids in membranes typically form a structure with two layers; hydrophilic heads outwards, hydrophobic fatty acid tails inwards. But in pollen grains, a large portion (40%) of the membrane lipids are non-bilayered, and occur in arrangements appearing as protuberances on the membrane surface (4, 5). Non-bilayer lipids support the dynamic organization of cellular membrane systems and have also been suggested to have an effect on membrane proteins (6, 7).

A stable and chemically resistant layer of sphingolipids protects the outer surface of the bilayered plasma membrane. Sphingolipids compose an estimated ∼40% of the total lipids in plasma membrane of plants and are also abundant lipid components of other endomembranes (8). Their backbone is an acylated amino alcohol (a LCB), with fatty acids, generally composed of 14–26 carbon atoms, linked to the amino group. This structure is called a ceramide. It can be modified through changes in chain length, methylation, hydroxylation and/or degree of desaturation of both the alcohol and the fatty acid (9). With further addition of a phosphate or a sugar residue to the alcohol, it can be varied into a plethora of different molecules. The biosynthesis pathway and metabolism of sphingolipids is ubiquitous and highly conserved among eukaryotes, but in plants, they vary more in structure than in other groups (10). Sphingolipids are central to many essential processes in plants including, but not limited to, pollen development. Deficient mutants have been shown to lack a surrounding intine layer (11). They are important in the response to biotic and abiotic stress, such as drought. Presumably, sphingolipids play a role during pollen dehydration. It is likely that stress resistance is due to the fatty acid and long-chain base composition (10).

Glycolylceramides, with headgroup hexoses of either glucose or mannose, are common in the intracellular membranes of pollen grains, relative to leaves. Simple sphingolipid metabolites, such as ceramide and free LCBs, have been shown to be important mediators in signaling cascades involved in various processes such as stress responses, the regulation of cell growth, differentiation, senescence, and apoptosis; in mammals notably also in inflammation. Apart from through the *de novo* pathway, can these metabolites be formed through the hydrolysis of complex sphingolipids. Ceramides and free LBCs can also be phosphorylated through various enzymes (12).

Plastids are double membrane-bound organelles with their own DNA. They originate from proplasts that may differentiate to fulfill various functions, i.e., to be chloroplasts, the site of photosynthesis. In pollen grains, photosynthesis is not necessary, but in the cytoplasm of the vegetative cell, there are usually numerous plastids that accumulate starch. In plastid membranes,

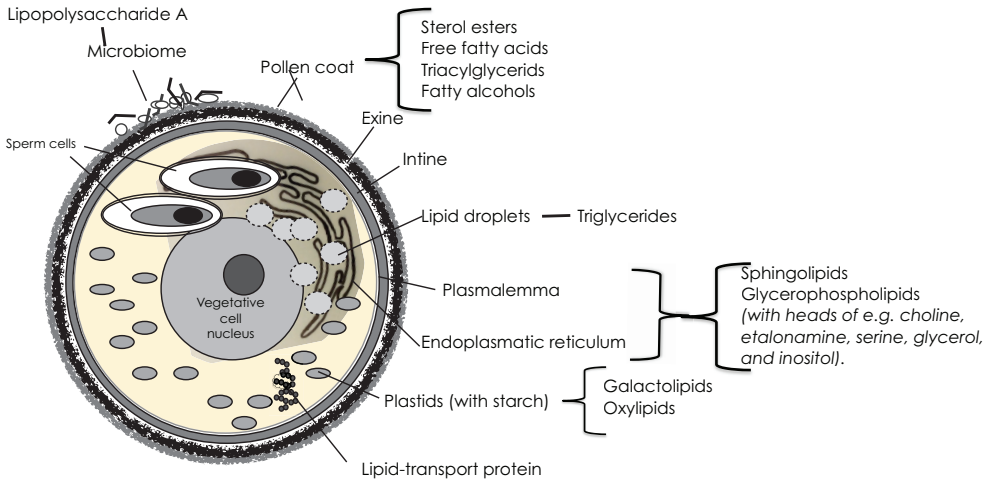


FIGURE 1 | A schematic image of a trinucleate angiosperm pollen grain with the different features that may contribute lipids with a potential impact on allergy; and where the possible candidate lipids are located.

galactolipids are more or less dominant. They contain high proportions of the fatty acids octadecadienoic (linoleic; 18:2, *cis*-9,12), octadecatrioneic acid (α-linolenic; 18:3, *cis*-9,12,15) acid, and hexadecatrienoid (roughanic; 16:3 *cis*-7, 10. 13) acid, which under situations of oxidative stress or through enzymatic action can be oxygenated to yield oxylipins (13). Unsaturated fatty acids (in *cis* formation) have "kinks" in their molecular structure, which prevent them from packing as closely as their saturated counterparts. This also makes them susceptible to oxidation (2). In mammals, oxidation catalyzes the generation of oxylipids involved in inflammation. In plants, oxylipins are involved in growth and development, as well as in defense and protection from many abiotic stresses. One example is the phytoprostranes that form from free-radical-catalyzed oxidation to yield oxygen radicals. Singlet oxygen, generated in chloroplasts during photosynthesis, is the most important ROS involved, but also superoxide anion radicals and hydrogen peroxide are involved (14). The result is a chain reaction, leading to the accumulation of hydroperoxides. If they include more than two double bonds, they can be further oxidized to yield many unstable molecules that have a prostaglandin G-ring system and are biologically active. They are powerful gene activators, especially for enzymes involved in the response to challenges by external conditions (15).

Lipid Droplets

The endoplasmatic reticulum enfolds lipid droplets with a diameter in the range of 0.5–2.0 μm. In pollen grains, they mainly contain triacylglycerides, but also 2–3% phospholipid, probably in a monolayer around the surface of the oil body. The chain lengths of the fatty acids in naturally occurring triglycerides vary, but most contain 16, 18, or 20 carbon atoms. The triacylglycerides accumulate throughout pollen maturation and about 20 % are used during this process (16).

Lipid-Transferring Proteins

The vegetative cell contains many lipid-binding proteins that contain hydrophobic binding sites for lipid ligands, including triacylglycerols and phospholipids. Most of these proteins will be secreted on the surface of the pollen grain. Although the *in vivo* functions of lipid-binding proteins (LTPs) remain unclear, accumulating evidence suggests a role in the transfer and deposition of monomers required for assembly of waterproof lipid barriers—such as cuticular wax and sporopollenin (17). They are necessary for pollen adhesion and germination on the stigma. Other roles are signaling during pathogen attacks and tolerance to abiotic stresses. LTPs bind both saturated and unsaturated fatty acyl chains. Furthermore, many can fit two fatty acyl chains in their cavity. Some LTPs are reported to bind to hydroxylated acyl chains (18, 19).

The Intine Is Produced by the Gametophyte

The intine surrounds the vegetative cell, beneath the exine. It consists of fibrillar cellulosa, hemicellulosa and pectine, with associated enzymes that act during germination and pollen tube growth. It develops after the exine. Part of the pectine layer becomes the precursor of the pollen tube wall. The intine is usually thicker under the apertures (20).

In the gymnosperm families Cupressaceae (e.g., *Cupressus* and *Juniperus*), Taxaceae (*Taxus*), and Taxodiaceae (e.g., *Sequoia*), which have inaperturate or monoaperturate pollen, the intine is very thick as compared to the exine and to the diameter of the pollen grain. In contact with water, it swells to rupture, casting off the fragile exine.

The Outer Wall of the Pollen Grain Is of Sporophytic Origin

The Exine

The exine usually consists of two layers [**Figure 2**; (21)]. The inner one is the endexine, which is a homogenous structure,

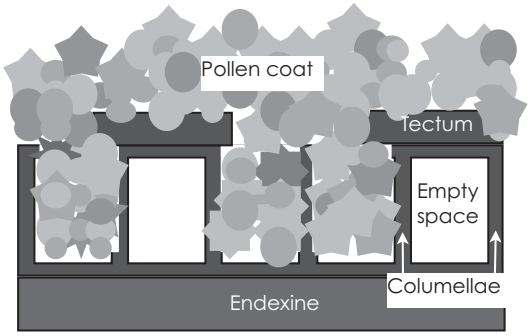


FIGURE 2 | The outer wall of the pollen grain is called the exine, and consists of the very resistant and inert compound sporopollenin. The exine is generally bilayered, with a lower and homogenous endexine and an upper and structured ectexine. The ectexine, in turn, has a foot layer, a layer of rods (columellae) and an outermost tectum. The rods are usually not tightly joined, but are separated by empty space. If the tectum does not cover the lower strata completely (is semitectate), the spaces can be filled with the sticky pollen coat. In insect-pollinated species, the pollen coat is ample and covers the surface of the pollen grain; in wind-pollinated species with intectate pollen, it may fill the spaces between the rods, but be lacking on the surface.

except for at the apertures. The outer one is the ektexine, which in its complete form has three layers. The lowermost is the foot layer and the outermost is the tectum; the middle layer consists of radially arranged rods (aka columellae), separated by empty space. Variation is large with regard to the thickness, sculpturing and arrangement of the rod layer, as well of the texture and integrity of the tectum. Although consisting of the extremely resistant and complex compound sporopollenin, the exine is not impermeable. Rowley et al. (22) identified numerous radial microchannels, ∼25 nm in diameter in mature pollen, that allow for a flow of water and for diffusion of small molecules into spaces between the rods and to the surface coating.

The exine is formed during early microspore life, initially through the contribution of its cytoplasm, but later and above all through accretions by the inner anther wall cell layer, the tapetum, part of the sporophyte.

In most pollen grains, there are thinner parts of the exine, termed apertures, through which the pollen tube generally emerges at germination. They also serve in the accommodation of volume changes at desiccation and hydration (23).

The exine is an extremely stable structure. Its main component is sporopollenin. Its insolubility has been an obstacle to in-depth analysis and its composition and biosynthesis is not fully understood. However, it appears to be a biopolymer of phenylpropanoid and lipidic monomers, such as very long-chained fatty acids and their polyhydroxylated derivatives (24).

The Pollen Coat

The outermost layer of the pollen wall, the pollen coat, is of a sticky nature and accumulates in depressions and spaces between the bacula [**Figure 2**; (25)]. It is mainly composed of lipids and is extremely hydrophobic. In addition, there are also proteins, pigments, aromatic substances and small molecules such as sugars (26). Lipids protect pollen grains against UV light damage, dehydration, and attack by pathogens. They play a key role in pollen-pistil interactions and in pollination by insects or other animals (27). Pollen from anemophilous plants generally contain less of the sticky pollen coat material than those from zoophilous species. This trend is evident in genera where there is a transition from zoophily to anemophily, such as *Fagopyrum* and *Castanea*. In contrast to the case of zoophilous pollen, where there is a sticky layer on the exine surface, it is deposited between the columellae and inside exine cavities in pollen generally dispersed by the wind (28).

Just as the exine, do the contents of the pollen coat mainly emanate from the tapetal cells, after their apoptosis. The coat is formed from their debris (29). The lipid composition of the pollen coat is completely different from that in other parts of the pollen grain.

Sterol esters

The relative dominance of sterol esters and fatty acids in the pollen coat appears to vary. Sterol esters contain relatively saturated acyl groups, and may function in the maintenance of the pollen coat in a semi-solid form that enclose and keep the embedded proteins and other substances in place (25). Sterols differ in the length of the side chain and the number and positions of double bonds and attached methyl groups. The sterols found in most tissues of higher land plants mainly consist of sitosterol, stigmasterol, campesterol, and to a lower amount of cholesterol. In pollen the composition is different and more complex. It varies between closely related species, which may be due to variation in pollination system: many pollinating insects lost the ability to synthesize squalene and depend on the uptake from sterols from their nutrition. In a study of pollen from 22 allergenic species (30), β-sitosterol, [(3b-,24R)-ergost- 5-en- 3-yl]oxy], and stigmastan-3,5-diene dominated among sterols, identified in 12–16 pollen species. In addition, stigmasterol and the tripene β-amyrin were present in 6 and 5 species, respectively, of the pollen analyzed. In another study, the major sterol ester in *Brassica napus* was found to be campestdienol, followed by stigmasterol, campesterol and sitosterol (3).

Fatty acids

In their study of pollen from 22 allergenic species, Bashir et al. (30) found that saturated and unsaturated fatty acids had the highest concentration and abundance. The dominating polyunsaturated fatty acid was octadecadienoic (linoleic) acid (18:2, *cis*-9,12), followed by octadecatrieonic acid (α-linolenic;18:3, *cis*-9,12,15), but there was a large variation among the species. Among saturated fatty acids, the six most abundant were hexadecanoic (palmitic; 16:0), tetradecanoic (myristic; 14:0), eicosanoic (arachidic; 20: 0), docosanoic (behenic; 22:0), heptadecanoic (margaric; 17:0), and nonanoic (pelargonic; 9:0) acid. Octadecanoic (stearic; 18:0) acid was present in six species, and there were also others with fewer occurrences (30). The ratio between unsaturated and saturated fatty acids varies between species. Both kinds often occur as triacylglycerides in the pollen coat (31).

Very long chain lipids contribute to the hydrophobic cuticle on the surface of all higher plants; their abundance in the pollen

coat, however, varies among species. Bashir et al. (30) found 25 different saturated, normal, and branched- chain hydrocarbons with between 6 and 35 carbon atoms. The odd-numbered series of 27, 29, 23, 17, and 21 carbon atoms were the most abundant. Long-chain n-alkanes 31:0 were detected in all grasses, but were rare in other samples. This was also true for 24:0.

Bashir et al. (30) also found significant differences among species with regard to dicarbyxolic acids, with (Z)-butenendioic, butanedioic, and buteneioic (E) acids dominating. They also identified alkenes, fatty alcohols, mono-unsaturated alcohols, and aldehydes.

The Pollen Microbiome Contributes to the Lipidome

The sticky pollen coat, containing sugars and lipids, is a favorable habitat for microorganisms. Both bacteria and fungi have been observed on the pollen surface (**Figure 1**), although the knowledge of their diversity still is quite new. Heydenreich et al. (32) demonstrated that grass pollen grains contained high numbers of Gram- negative and Gram-positive species. Ambika Manirajan (33) found that Proteobacteria was the dominant phylum in all pollen species, followed by Actinobacteria, Acidobacteria, and Firmicutes. Both plant species and pollination type significantly influenced structure and diversity of the pollen microbiota. Variation between species was significantly smaller between insect-pollinated species as compared to the wind-pollinated ones, suggesting an equalizing effect by insect vectors.

The outer membrane of Gram-negative bacteria is dominated by lipopolysaccharide (LPS; aka endotoxin), which is important, e.g., for the structural integrity of the bacteria, resistance to chemical attack, and interaction with predators. LPS can be toxic, due to the component Lipid A, which is a phosphorylated glucosamine disaccharide with multiple fatty acids. Gram-positive bacteria have only one membrane, surrounded by a thick peptidoglycan layer.

THE POLLEN GRAIN IN ACTION

When the pollen grain lands on a stigma in a flower, it must adhere to its surface. The exine structure then plays a significant role, as do compounds in the pollen coat, not least the lipids (34). When they arrive, most pollen grains generally contain little water. In most species, they are partially dehydrated until 15–35 %, just before or after anther opening, and quiescent. A number of species, e.g., of Urticaceae and Poaceae, remain partly hydrated at dispersal (35). In order to fulfill their function, i.e., fertilization, all pollen grains must be rehydrated, germinate and produce a pollen tube.

Stigmas could be "wet" or "dry," depending on the absence or presence of secretions during the receptive period (36, 37). In species with the wet type of stigma, pollen grains are immediately surrounded by the watery exudates, and are captured due to their stickiness and surface tension (38). In dry stigmas, the surface cells are intact and covered with a cell wall, a waxy cuticle, and a proteinaceous pellicle. Mobilization of pollen coat leads to mixing of lipids and proteins and formation of a "foot" of contact on the stigma surface (39). Thereby, secretion of lipid-rich material is further enhanced, leading to movement of water from the stigmatic papillae to the pollen. This interaction is facilitated by the degradation of lipids by lipases in the pollen coat and pollen wall, as well as by proteins. It leads to swelling of the pollen grain, and the emergence of a pollen tube from one of the apertures. The tube is directed toward the stigma by the gradient of an increasing water potential, which also causes polarization of the pollen cytoplasm. Unsaturated triacylglycerides are required in this process and necessary for the penetration of the stigma (31).

Hydration transforms the non-polar vegetative cell in the pollen grain to be highly polarized within minutes. The sphingolipid glycosyl inositol phosphorylceramides (GPICs) are very common components of plant membranes, but was said to be less abundant in pollen (3). However, in Arabidopsis GPIC-deficient mutants, pollen tubes behaved abnormal, and were not able to properly navigate or communicate with female tissues after pollination, indicating that GPICs indeed are important for the function of the pollen grain. Disruption of sphingolipids containing very long fatty acid chains is known to cause mislocalization of polar membrane proteins (40).

When the pollen grain still is dehydrated, the lipid droplets in the cytoplasm that were not consumed during pollen maturation, are enfolded in a network of the endoplasmatic reticulum. In olive (*Olea europea*) they were observed to enter the emerging pollen tube and to be degraded and apparently be converted directly into membrane lipids (16). In addition, lipid droplets are synthesized throughout the life of the pollen tube, in plasmids present in the vegetative cell cytoplasm that entered the pollen tube. Growth is usually rapid, so the demand for membrane lipids is substantial. It was suggested that sterols also provide necessary membrane material, but rather, they play a role during subcellular processes underlying polar tube tip growth. The composition of sterols changes during pollen tube growth. The change is suggested to promote maximal tube elongation (3).

The *Arabidopsis* alkaline ceramidase TOD1 is a key turgor pressure regulator in plant cells. Turgor pressure plays pivotal roles in the growth and movement of plant cells; thus also in pollen tubes. Li-Yu Chen et al. (41) demonstrate that *Arabidopsis* mutants lacking an alkaline ceramidase have a higher turgor than the wild type and show growth retardation.

POLLEN LIPIDS IN ALLERGY

When bioaerosols (bacteria, fungi, pollen etc.) come into contact with human tissues facing the external environment, they will meet with so called antigen-presenting cells (APCs), e.g., dendritic cells (DCs). APCs recognize and process antigens and then migrate from the peripheral tissues to a lymph node, where they present the antigen on the cell surface of naive T cells. The dendritic cells also secrete small proteins—cytokines—that influence the polarization of T cell development into different subsets, called effector cells, that induce antibody production and transformation of other cells. The aim is to eliminate the antigen and resolve the infection. After the initial immune response, most of the effector cells die, while a fraction survive and become

long-lived memory cells. The host is then said to be sensitized. At future contact with the antigen, he or she will react.

Mature and polarized T cell subsets react to different antigens. Th1 cells react to bacteria, viruses and other intracellular pathogens, and eliminate cancerous cells. In focus of the present review are so called Th2 cells, which have evolved as a response to helminths, but also are activated by allergenic proteins. Dendritic cells stimulate T cells into this pathway through the cytokine IL-4, which in turn also secrete IL-4, as well as IL-13. Thereby, they activate B cells to produce specific IgE-antibodies, They also secrete IL-5, which increases the production of eosinophils in the bone marrow. Eosinophils are lymphocytes containing toxic cytoplasmic granulae. High levels of eosinophils lead to inflammation and tissue damage.

When the sensitized person reencounters an allergen, B-cells proliferate and produce IgE antibodies. These bind to mast cells and cross-link to the allergen, thereby activating the mast cells, which start to release granule proteins, as well as different mediators that provoke the allergic reaction. Mast cells, T cells and macrophages produce cytokines that recruit eosinophils and T cells to the inflammatory site (42).

Natural killer T cells are highly conserved cells, that play an important role at the mucosal surfaces as part of the first line of defense of the immune system. They are present in peripheral tissues and, after activation, display effector functions through the rapid secretion of cytokines, including those involved in a Th2- reaction (43).

Recent discoveries have led to the identification of a novel group of immune cells, the innate lymphoid cells (ILCs). The members of this group are divided into three subpopulations. The subset ILC2s provides a source of the Th2 cytokines, IL-4, IL-5, and IL-13, upon activation by epithelial cell-derived cytokines and lipid mediators (leukotrienes and prostaglandin), and promote structural and immune cell responses in the airways after antigen exposure. Their response appears to be independent of T and B cells (44).

Antigen-Presenting Cells Carry Lipid-Binding Receptors

Toll-like receptors (TLRs) are a class of proteins that are expressed on the membranes of several cells both in the innate and in the adaptive immune system, as well as of non-immune cells such as in the airway epithelium. TLRs recruit proteins that start a chain reaction, ultimately leading to upregulation or suppression of genes that orchestrate inflammatory responses and other transcriptional events. They recognize molecules that are broadly shared by potentially pathogenic organisms and foreign molecules, which deviate from molecules that belong to the host.

TLR-4 and TLR-2 recognize lipids. TLR-4 recognizes LPS from Gram-negative bacteria, and then interacts with three different extracellular proteins, LPS-binding protein (LBP), CD14 and, myeloid differentiation protein 2 (MD-2). TLR-2 recognizes a wide range of compounds from Gram-positive and Gram-negative bacteria, as well as from mycoplasma and yeast. In both cases, the result is proinflammatory cytokine production (45). Environmental LPS exposure of individuals with allergic airway diseases, including asthma, is known to exacerbate the disease (46).

The peroxisome proliferator-activated receptors (PPARs) are a group of nuclear receptor proteins that are associated with lipids. When the PPAR binds a ligand, transcription of target genes is increased or decreased, depending on the gene. PPARγ is important in asthma, allergy, and airway inflammatory responses through up-regulation of PTEN [phosphatase and tensin homolog; (30)]. Among naturally occurring agents that directly bind with and activate PPARγ are various polyunsaturated fatty acids, which are common in pollen grains, in a free form or as constituents in phospholipids, glycolipids, and triacylglycerol acyl chains, as well as arachnoids, which may occur as oxylipids.

Lipids Bind to CD1 Molecules

Lipids and glycolipids are recognized by CD1 (cluster of differentiation) molecules, which constitutes a family of glycoproteins, that are expressed on various mammal immune cells and on planar membranes such as in the airway epithelium. They are structurally similar to MHC class I molecules that bind peptids, but are generally not polymorphic.

There are four different CD1 proteins with their own unique way to bind lipids, as their antigen-binding pockets differ. CD1d is expressed on professional APCs including dendritic cells (DCs), macrophages (M), monocytes, and some B cells. CD1d molecules activate a certain subset of NKT cells called "invariant NKT" (iNKT), since they are restricted to CD1d. They were first regarded as a part of the defense against lipids of microbial origin, However, a number of other exogenous and endogenous ligands could be presented by CD1d to iNKT cells, including polar lipids, diacylglycerols, free fatty acids, and triacylglycerols isolated from pollen grains (47, 48).

Dendritic cells are able to recognize and uptake lipids through their TLR-receptors, or through PPAR. Abos-Gracia et al. (48) exposed monocyte-derived DCs to olive pollen grains in an *in vitro*-culture system (**Figure 3**). The pollen grain was surrounded and enclosed by the DCs, followed by their maturation and display of an increased CD1d expression, as a result of activation of the PPAR receptor. Abos-Gracia et al. (48) showed that polar lipids from olive pollen (including phospholipids and glycolipids) indeed were able to increase CD1d surface expression. In an earlier study, (49, 50) showed that cloned gδ T lymphocytes from subjects with allergy, but not normal controls, recognized *Cupressus* and *Olea* pollen-derived phosphatidyl- ethanolamine (PE) in a CD1d-restricted fashion. Only 16:0/18:2 and 18:2/18:2 PE were stimulatory. There was no response from disaturated PE, phosphatidylcholine, neutral lipids, or protein extract.

In their assay of lipids occurring on the surface of pollen grains, Bashir et al. (30) stimulated dendritic cells and NKT-cells from two strains of mice with various kinds of lipids that have been related to allergic reactions. DCs and NKTs appeared to engage in an activation loop, reciprocally stimulating the production of Th2 cytokines **Figure 3**. One of the mouse strains lacked the MyD88 gene, which provides instructions for making a protein involved in signaling within immune cells. The MyD88 protein acts as an adapter, in particular

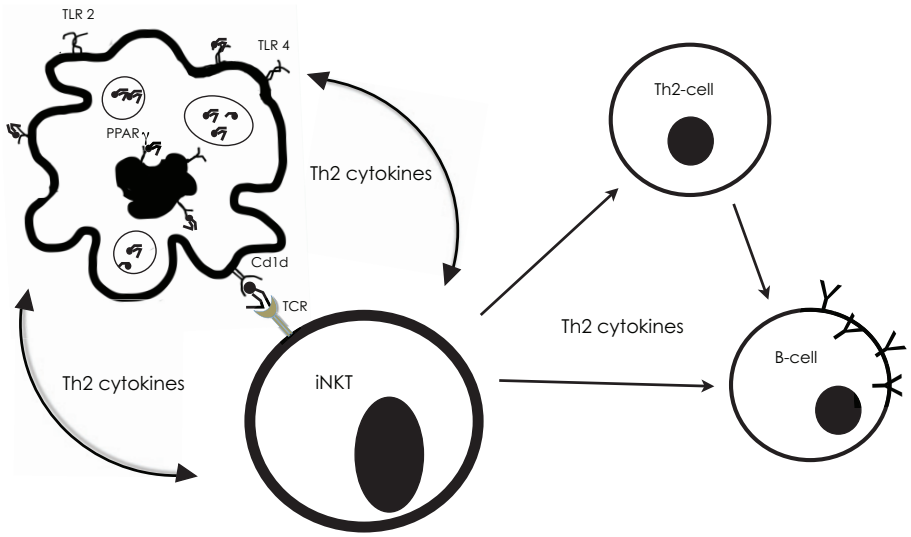


FIGURE 3 | The interplay between antigen-presenting cells (APC), invariant NKT cells, Th2-cells and B-cells, with examples from what is described from reactions to pollen lipids. The Toll-like receptors 2 and 4, on the surface of APCs, as well as the nuclear receptor PPARg in their interior, can bind to lipids, which then are presented by Cd1d molecules on their surface. Then, the Cd1d-bound lipid can be recognized by T cell receptor (TCR) on the surface of the iNKT. Simultaneously, the APC produces cytokines, which skew cytokine production in the iNKT to promote a Th2-inflammatory response. The APC will in turn be stimulated by the iNKT cytokine production, and they will engage in an activation loop, reciprocally stimulating the production of Th2 cytokines (e.g., IL-4, IL-5, IL-13).

transferring signals from Toll-like receptors and interleukin-1 (IL-1) to the proteins that relay signals inside the cell. The parallel study of cells from wild type and deficient mice would illuminate the importance of these factors for the response to lipid exposure. The results showed that the MyD88 pathway was necessary for the induction of a proinflammatory response to saturated fatty acids, n- alkanes, aliphatic alcohols, and sterols, with some exceptions—and then only in the presence of NKT-cells.

The cytokine IL-13 could induce the secretion of Th2-promoting factors from DCs, further stimulating iNKT-cells to produce more of the Th2-cytokines. The fatty acids eicosanoic acid (20:0) and docosanoic acid (22:0) caused rapid, substantial, and specific up-regulation of Th2-associated IL- 13 from MyD88 deficient DCs/NKT. This result suggested the presence of toll-like receptor (TLR)-independent pattern-recognition receptor in Th2—induction against eicosanoic acid and docosanoic acid. Expression of IL-13 was stimulated also by the n-alkane with 24 carbon atoms and by the aliphatic alcohols 1- tetradecanol and 1-octacosanol (30).

Tumor necrosis factor (TNF, tumor necrosis factor α, TNF α, cachexin, or cachectin) is a cytokine involved in systemic inflammation and is one of those that make up the acute phase reaction. Furthermore, TNF-α might be important in the recruitment of eosinophils to the allergic inflammatory site. Bashir et al. (30) found that the saturated fatty acids 17:0–26:0 induced TNF-α, in DCs from the wild-type mice, with even more enhanced excretion in the presence of NKT-cells. The greatest release was induced by tetracosanoic (24:0) and docosanoic (22:0) acids, followed by hexacosanoic (26:0), and heptadecanoic (17:0) acids. Also n- alkanes, especially with 25 carbon atoms, and aliphatic alcohols, especially 1-tetradecanol, and the sterol b-sitosterol stimulated TNF-α.

Disintegration of pollen may enhance the bioavailability of lipid compounds from inside the pollen grain. As mentioned above, the pollen grains in a number of gymnosperm families (Cupressaceae, Taxaceae, Taxodiaceae) have an exceptionally thick intine and a thin exine. At pollination, the pollen grains are captured in a liquid secreted from the micropyle. The hydrophilic and elastic intine then swells, causing the non-elastic exine to burst and be shed, leaving the intine exposed. After 10–15 min, the expanding intine starts to break, and the cytoplasmic components disperse in the liquid. Exposed acyl side chains of the non-bilayer phospholipids that protrude from membrane surfaces [(5); section The Vegetative Cell of the Gametophyte] may enter the pocket of a CD1 molecule on a DC, making the contact between pollen grain and the DC-cell stable and activating iNKT and other CD1-presenting T cells. Then, DCs migrate into draining lymph nodes, where allergenic proteins are presented to conventional lymphocytes (T- and B-cells), which amplify the allergic reaction (3; 39).

Activation and up-regulation of CD1d by pollen-derived lipids also occur in other types of APCs, such as macrophages and monocytes, apart from in dendriti cells. Both macrophages and monocytes, treated with olive pollen lipids showed an increase in CD1d gene expression (47).

Lipids Are Ligands to Allergenic Proteins

The allergenic potential of non-specific lipid-binding proteins have been attributed both to their low molecular mass and to their high thermal and proteolytic stability, which allow them to reach the immune system in a biological intact form.

But their binding to different types of lipids, including fatty acids, phospholipids, glycolipids, and prostaglandin B, could contribute to the activation of innate immune cells, and enhance an IgE dominant response (51). nsLTP proteins are very versatile with regard to possible ligands. The volume of their internal hydrophobic cavities are sufficient to accommodate either single- or double-chain lipids bound in different modes. It is not yet known how these variants affect the allergenic potency. The possible adjuvant nature of the lipid ligands, when combined with the allergen, must be further evaluated. Lipid-binding to nsLTPs was shown to result in CD1d- restricted activation of iNKT cells promoting allergic sensitization. There might also be other mechanisms, where lipids engage pathogen recognition receptors (PRRs) on the surface of immune and epithelial cells, or influence the absorption of allergens through the epithelial barrier (52).

The major allergen of Brazil nut (*Bertholletia excelsa*), Ber e 1, requires association to a specific fraction of the lipids present in the nuts, in order to trigger allergic Th2 responses in mice and humans (53). This fraction contains neutral and common phospholipids. Whereas the allergen alone is not able to induce sensitization, the complex of allergen and lipid promptly promoted high levels of Ber-specific IgG1 as well as total IgE. It was found that Ber e 1 has a lipid-binding pocket similar to the LTP-binding pocket in wheat protein, and results indicated interaction between the allergen and lipids in the aforementioned fraction. Furthermore, the complex allergen-lipid induced the Th2-associated cytokine IL-4-production by splenic iNKT-cells of mice, leading to the production of anaphylactic antibodies, whereas mice lacking iNKT did not react in this way. Although Ber e 1 is not a pollen-, but a seed-associated allergen, this case illuminates how lipids can enhance the allergenicity of a protein, and the role of iNKT in the provocation of an allergic reaction. Among other components in the active lipid fraction where triclycerides, sterols, and phosphatidylethanolamine, all present in pollen; the latter also reported to be recognized by T-lymphocytes in a Cd1d-restricted fashion in a Olea pollen extract (49).

The natural ligand of the main isoform of the major birch pollen protein Bet v 1 is quercitin, which binds to iron when ligated to the protein. When iron is present, Bet v 1 has no potential to provoke an Th2 response, but is it absent, it manipulates T cells toward a Th2 polarization (54, 55). The ligand interaction increases the volume of the hydrophobic pocket, causing a structural change that might have an impact on the uptake and processing of the protein (56, 57).

Pollen-Associated Lipid Mediators

Human omega-6 polyunsaturated fatty acid-derived eicosanoids, particularly leukotrienes and prostaglandins, regulate chronic type 2 inflammation on multiple levels, as being released by mast cells, macrophages, DCs, and endo- and epitheliar cells. Upon hydration, pollen grains rapidly release significant amounts of lipids- the so-called pollen-associated lipid mediators (PALMs) that show structural and functional homology to eicosanoids (58). One group is homologous with leukotriene B. They are able to recruit human neutrophils and eosinophils, also in non-sensitized individuals. E1- phytoprostanes are similar to human prostaglandins, sharing the characteristic ring systems. They induce maturing and migration of DCs, and cause them to prime naive T cells to Th2 responses, while they will dampen or inhibit the induction of Th1 responses.

CONCLUSION

It is evident that an understanding of atopic reactions to pollen compounds must involve knowledge about the impact of pollen-derived lipids and their ability to activate specialized receptors that start a chain reaction, which "skews" the immune system toward a so called Th2-reaction.

The human nasal mucosa and the conjunctiva can be exposed to potentially allergy-promoting lipids present at the surface of the pollen grain, such as sterols and free fatty acids in the pollen coat, or delivered there from the interior of the grain, such as lipids bound to proteins, and phytoprostranes mimicking mammal lipid mediators. In *Cupressus*, it was shown that hydration of the pollen grain causes disintegration and exposure of membrane-bound phospoholipids, which contribute to allergic reactions. Pollen rupture and exposure of cytoplasmic debris, including possible fragments of ER, plasmalemmas, plastid membranes, and lipid droplets, which are sources of glycophospholipids, sphingolipids, triacylglycerides, and their derivatives, is not unique for cupressacean pollen, but also frequently occurs in that from angiosperms. In contrast to intact pollen grains, these "sub-pollen particles" are as small as to be respirable and may penetrate into the lower airway, where severe allergic reactions including asthma attacks could be triggered (59, 60). Hence, the bioavailability of pollen-derived lipids is substantial and should be further considered.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

REFERENCES

1. D'Amato G, Cecchi L, Bonini S, Nunes C, Annesi-Maesano I, Behrendt H, et al. Allergenic pollen and pollen allergy in Europe. *Allergy* (2007) 62:976–90. doi: 10.1111/j.1398-9995.2007.01393.x
2. Fahlberg P. *Plant Oxylipins and Lipid Transfer Proteins in Defense—It is all About the Fat*. Dissertation, University of Gothenburg (2017).
3. Ischebeck T. Lipids in pollen — They are different. *Biochimica et Biophysica Acta* (2016) 1861:1315–28. doi: 10.1016/j.bbalip.2016.03.023
4. Agea E, Russano A, Bistoni O, Mannucci R, Nicoletti I, Corazzi L, et al. Human CD1-restricted T cell recognition of lipids from pollens. *J Exp Med*. (2005) 202:295–308. doi: 10.1084/jem.20050773
5. Chichiriccò G, Pacini E. Cupressus arizonica pollen wall zonation and *in vitro* hydration. *Plant Syst Evol*. (2008) 270:231–42. doi: 10.1007/s00606-007-0610-6

6. Frolov VA, Shnyrova AV, Zimmerberg J. Lipid polymorphisms and membrane shape. *Cold Spring Harb Perspect Biol.* (2011) 3:a004747. doi: 10.1101/cshperspect.a004747
7. van den Brink-van der Laan E, Antoinette Killian J, de Kruijff B. Nonbilayer lipids affect peripheral and integral membrane proteins via changes in the lateral pressure profile. *Biochimica et Biophysica Acta (BBA)* (2004) 1666:275–88. doi: 10.1016/j.bbamem.2004.06.010
8. Luttgeharm KD, Kimberlin AN, Cahoon RE, Cerny RL, Napier JA, Markham JE, et al. Sphingolipid metabolism is strikingly different between pollen and leaf in *Arabidopsis* as revealed by compositional and gene expression profiling. *Phytochemistry* (2012) 115:121–9. doi: 10.1016/j.phytochem.2015.02.019
9. Pata MO, Hannun YA, Ng CKY. Plant sphingolipids: decoding the enigma of the Sphinx. *New Phytol.* (2010) 185:611–30. doi: 10.1111/j.1469-8137.2009.03123.x
10. Michaelson LV, Napier JA, Molino D, Faure JD. Plant sphingolipids: their importance in cellular organization and adaption. *Biochimica et Biophysica Acta* (2016) 1861:1329–35. doi: 10.1016/j.bbalip.2016.04.003
11. Dietrich CR, Han G, Chen M, Berg RH, Dunn TM, Cahoon EB. Loss-of-function mutations and inducible RNAi suppression of Arabidopsis LCB2 genes reveal the critical role of sphingolipids in gametophytic and sporophytic cell viability. *Plant J.* (2008) 54:284–98. doi: 10.1111/j.1365-313X.2008.03420.x
12. Bartke N, Hannun YA. Bioactive sphingolipids: Metabolism and function. *J Lipid Res.* (2009) 50:S91–6. doi: 10.1194/jlr.R800080-JLR200
13. Mosblech A, Napier JA, Molino D, Faure JD. The critical requirement for linoleic acid is pollen development, not photosynthesis, in an Arabidopsis mutant. *Plant Cell* (2009) 8:403–16. doi: 10.1105/tpc.8.3.403
14. Triantaphylidès C, Krischke M, Hoeberichts FA, Ksas B, Gresser G, Havaux M, et al. Singlet oxygen is the major reactive oxygen species involved in photooxidative damage to plants. *Plant Physiol.* (2008) 148:960–8. doi: 10.1104/pp.108.125690
15. Durand T, Bultel-Poncé V, Guy A, Berger S, Mueller MJ, Galano JM. New bioactive oxylipins formed by non-enzymatic free-radical-catalyzed pathways: the phytoprostanes. *Lipids* (2009) 44:875–88. doi: 10.1007/s11745-009-3351-1
16. Zienkiewicz A, Zienkiewicz K, Rejón JD, Rodríguez-García MI, Castro AJ. New insights into the early steps of oil body mobilization during pollen germination. *J Exp Bot.* (2013) 64:293–302. doi: 10.1093/jxb/ers332
17. Edqvist J, Blomqvist K, Nieuwland J, Salminen TA. Plant lipid transfer proteins: are we finally closing in on the roles of these enigmatic proteins? *J Lipid Res.* (2018) 59:1374–82. doi: 10.1194/jlr.R083139
18. Suárez-Cervera M, Vega-Maray A, Castells T, Rodríguez-Rajo FJ, Asturias JA, Le Thomas A, et al. An approach to the knowledge of pollen and allergen diversity through lipid transfer protein localisation in taxonomically distant pollen grains. *Grana* (2008) 47:272–84. doi: 10.1080/00173130802513776
19. Salminen TA, Blomqvist K, Edqvist J. Lipid transfer proteins: classification, nomenclature, structure, and function. *Planta* (2016) 244:971–97. doi: 10.1007/s00425-016-2585-4
20. Scott RJ, Spielman M, Dickinson HG. Stamen structure and function. *Plant Cell* (2004) 16:S46–60. doi: 10.1105/tpc.017012
21. Faegri K, Iversen J, Kaland PE, Krzywinski K. *Textbook of Pollen Analysis.* London: John Wiley and Sons (1989).
22. Rowley JR, Skvarla JJ, El-Ghazaly G. Transfer of material through the microspore exine - from the loculus into the cytoplasm. *Can J Botany* (2003) 81:1070–82. doi: 10.1139/b03-095
23. Pacini, E, Bassani M. Harmomegathic features of spores and pollen grains. *Giornale Botanico Italiano* (1988) 122:92–3.
24. Zhang D, Shi J, Yang X. Role of lipid metabolism in plant pollen exine development. *Subcell Biochem.* (2016) 86:315–37. doi: 10.1007/978-3-319-25979-6_13
25. Piffanelli P, Ross JHE, Murphy DJ. Biogenesis and function of the lipidic structures of pollen grains. *Sex Plant Reprod.* (1998) 11:65–80. doi: 10.1007/s004970050122
26. Wu SSH, Moreau RA, Whitaker BD, Huang AHC. Steryl esters in the elaioplasts of the tapetum in developing *Brassica* anthers and their recovery on the pollen surface. *Lipids* (1999) 34:517–23. doi: 10.1007/s11745-999-0393-5
27. Bublin M, Breiteneder H. Cross-reactivity of peanut allergens. *Curr Allergy Asthma Rep.* (2014) 14:426. doi: 10.1007/s11882-014-0426-8
28. Pacini E, Hesse M. Pollenkitt - Its composition, forms and functions. *Flora* (2005) 200:399–415. doi: 10.1016/j.flora.2005.02.006
29. Heslop-Harrison, J. (1968). Tapetal origin of pollen-coat substances in *Lilium*. *New Phytol.* 67:779–86. doi: 10.1111/j.1469-8137.1968.tb06395.x
30. Bashir MEH, Lui JH, Palnivelu R, Naclerio RM, Preuss D. Pollen lipidomics: lipid profiling exposes a notable diversity in 22 allergenic pollen and potential biomarkers of the allergic immune response. *PLoS ONE* (2013) 8:e57566. doi: 10.1371/journal.pone.0057566
31. Wolters-Arts M, Lush WM, Mariani C. Lipids are required for directional pollen-tube growth. *Nature* (1998) 392:818–21. doi: 10.1038/33929
32. Heydenreich B, Bellinghausen I, König B, Becker WM, Grabbe S, Petersen A, et al. Gram-positive bacteria on grass pollen exhibit adjuvant activity inducing inflammatory T cell responses. *Clin Exp Allergy* (2012) 42:76–84. doi: 10.1111/j.1365-2222.2011.03888.x
33. Ambika Manirajan B, Ratering S, Rusch V, Schwiertz A, Geissler-Plaum R, Cardinale M, et al. Bacterial microbiota associated with flower pollen is influenced by pollination type, and shows a high degree of diversity and species-specificity. *Environ Microbiol.* (2016) 18:5161–74. doi: 10.1111/1462-2920.13524
34. Shakya R, Bhatla SC. A comparative analysis of the distribution and composition of lipidic constituents and associated enzymes in pollen and stigma of sunflower. *Sex Plant Reprod.* (2010) 23:163–72. doi: 10.1007/s00497-009-0125-0
35. Pacini E. From anther and pollen ripening to pollen presentation. *Plant System Evol.* (2000) 222:19–43. doi: 10.1007/BF00984094
36. Sharma B, Bhatla SC. Structural analysis of stigma development in relation with pollen-stigma interaction in sunflower. *Flora* (2013) 208:420–9. doi: 10.1016/j.flora.2013.07.003
37. Heslop-harrison Y, Shivanna KR. The receptive surface of the angiosperm stigma. *Ann Botany* (1977) 41:1233–58. doi: 10.1093/oxfordjournals.aob.a085414
38. Edlund AF, Swanson R, Preuss D. Pollen and stigma structure and function: the role of diversity in pollination. *Plant Cell* (2004) 16:S84–97. doi: 10.1105/tpc.015800
39. Zinkl GM, Preuss D. Dissecting *Arabidopsis* pollen-stigma interactions reveals novel mechanisms that confer mating specificity. *Ann Botany* (2000) 85:15–21. doi: 10.1006/anbo.1999.1066
40. Tartaglio V, Rennie EA, Cahoon R, Wang G, Baidoo E, Mortimer JC, et al. Glycosylation of inositol phosphorylceramide sphingolipids is required for normal growth and reproduction in *Arabidopsis*. *Plant J.* (2017) 89:278–90. doi: 10.1111/tpj.13382
41. Chen LY, Shi DQ, Zhang WJ, Tang ZS, Liu J, Yang WC. The Arabidopsis alkaline ceramidase TOD1 is a key turgor pressure regulator in plant cells. *Nat Commun.* (2015) 6:6030. doi: 10.1038/ncomms7030
42. Parham P. *The Immune System.* 3rd ed. New York, NY: Garland Publishers (2009).
43. Wingender G, Rogers P, Batzer G, Lee MS, Bai D, Pei B, et al. Invariant NKT cells are required for airway inflammation induced by environmental antigens. *J Exp Med.* (2011) 208:1151–62. doi: 10.1084/jem.20102229
44. Mazzurana L, Rao A, van Acker A, Mjösberg J. The roles for innate lymphoid cells in the human immune system. *Semin Immunopathol.* (2018) 40:407–19. doi: 10.1007/s00281-018-0688-7
45. Mittag D, Varese N, Scholzen A, Mansell A, Barker G, Rice G, et al. TLR ligands of ryegrass pollen microbial contaminants enhance Th1 and Th2 responses and decrease induction of Foxp3hi regulatory T cells. *Eur J Immunol.* (2013) 43:723–33. doi: 10.1002/eji.201242747
46. Williams CMM, Rahman S, Hubeau C, Ma HL. Cytokine pathways in allergic disease. *Toxicol Pathol.* (2012) 40:205–15. doi: 10.1177/0192623311430694
47. Gracia BA, López Relanõ J, Revilla A, Castro L, Villalba M, Adrados BM, et al. Human invariant natural killer T cells respond to antigen-presenting cells exposed to lipids from *Olea europaea* pollen. *Int Arch Allergy Immunol.* (2017) 173:12–22. doi: 10.1159/000467394
48. Abós-Gracia B, Del Moral MG, López-Relaño J, Viana-Huete V, Castro L, Villalba Martínez-Naves E. *Olea europaea* pollen lipids activate invariant natural killer T cells by upregulating CD1d expression on dendritic cells. *J Allergy Clin Immunol.* (2013) 131:1393–9 e1395. doi: 10.1016/j.jaci.2012.11.014

49. Russano AM, Agea E, Corazzi L, Postle AD, De Libero G, Porcelli S, et al. Recognition of pollen-derived phosphatidyl-ethanolamine by human CD1d-restricted γδ T cells. *J Allergy Clin Immunol*. (2006) 117:1178–84. doi: 10.1016/j.jaci.2006.01.001
50. Russano AM, Agea E, Casciari C, De Benedictis FM, Spinozzi F. Complementary roles for lipid and protein allergens in triggering innate and adaptive immune systems. *Allergy* (2008) 63:1428–37. doi: 10.1111/j.1398-9995.2008.01810.x
51. Del Moral MG, Martínez-Naves E. The role of lipids in development of allergic responses. *Immune Netw*. (2017) 17:133–43. doi: 10.4110/in.2017.17.3.133
52. Scheurer S, Schülke S. Interaction of non-specific lipid-transfer proteins with plant-derived lipids and its impact on allergic sensitization. *Front Immunol*. (2018) 9:1389. doi: 10.3389/fimmu.2018.01389
53. Mirotti L, Florsheim E, Rundqvist L, Larsson G, Spinozzi F, Leite-De-Moraes M, et al. Lipids are required for the development of Brazil nut allergy: the role of mouse and human iNKT cells. *Allergy* (2013) 68:74–83. doi: 10.1111/all.12057
54. Jensen-Jarolim E. Happy 25th birthday, Bet v 1. *World Allergy Organ J*. (2014) 7:14. doi: 10.1186/1939-4551-7-14
55. Roth-Walter F, Gomez-Casado C, Pacios LF, Mothes-Luksch N, Roth GA, Singer J, et al. Bet v 1 from birch pollen is a lipocalin-like protein acting as allergen only when devoid of iron by promoting Th2 lymphocytes. *J Biol Chem*. (2014) 289:17416–21. doi: 10.1074/jbc.M114.567875
56. Gómez-Casado C, Dïaz-Perales A. Allergen-Associated immunomodulators: modifying allergy outcome. *Arch Immunol Ther Exp*. (2016) 64:339–47. doi: 10.1007/s00005-016-0401-2
57. Kitzmuller C, Zulehner N, Roulias A, Briza P, Ferreira F, Fae I, et al. Correlation of sensitizing capacity and T-cell recognition within the Bet v 1 family. *J Allergy Clin Immunol*. (2015) 136:151–8. doi: 10.1016/j.jaci.2014.12.1928
58. Traidl-Hoffmann C, Mariani V, Hochrein H, Karg K, Wagner H, Ring J, et al. Pollen-associated phytoprostanes inhibit dendritic cell interleukin-12 production and augment T helper type 2 cell polarization. *J Exp Med*. (2005) 201:627–36. doi: 10.1084/jem.20041065
59. Taylor PE, Jacobson KW, House JM, Glovsky MM. Links between pollen, atopy and the asthma epidemic. *Int Arch Allergy Immunol*. (2007) 144:162–70. doi: 10.1159/000103230
60. Thien F, Beggs PJ, Csutoros D, Darvall J, Hew M, Davies JM, et al. The Melbourne epidemic thunderstorm asthma event 2016: an investigation of environmental triggers, effect on health services, and patient risk factors. *Lancet Planet Health* (2018) 2:e255–63. doi: 10.1016/S2542-5196(18)30120-7

Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 14 February 2019
doi: 10.3389/fimmu.2019.00122

Lipophilic Allergens, Different Modes of Allergen-Lipid Interaction and Their Impact on Asthma and Allergy

Uta Jappe[1,2], Christian Schwager[1], Andra B. Schromm[3], Nestor González Roldán[4], Karina Stein[5], Holger Heine[5] and Katarzyna A. Duda[4]*

[1] Division of Clinical and Molecular Allergology, Research Center Borstel, Leibniz Lung Center, Airway Research Center North, German Center for Lung Research, Borstel, Germany, [2] Interdisciplinary Allergy Outpatient Clinic, Department of Pneumology, University of Luebeck, Borstel, Germany, [3] Division of Immunobiophysics, Research Center Borstel, Leibniz Lung Center, Borstel, Germany, [4] Junior Research Group of Allergobiochemistry, Research Center Borstel, Leibniz Lung Center, Airway Research Center North, German Center for Lung Research, Borstel, Germany, [5] Division of Innate Immunity, Research Center Borstel, Leibniz Lung Center, Airway Research Center North, German Center for Lung Research, Borstel, Germany

OPEN ACCESS

Edited by:
Philippe Saas, INSERM U1098 Interactions Hôte-Greffon-Tumeur & Ingénierie Cellulaire et Génique, France

Reviewed by:
Edward Knol, University Medical Center Utrecht, Netherlands
Colin Matthew Fitzsimmons, University of Cambridge, United Kingdom
Jesus Perez-Gil, Complutense University of Madrid, Spain

***Correspondence:**
Uta Jappe
ujappe@fz-borstel.de

Specialty section:
This article was submitted to Inflammation, a section of the journal Frontiers in Immunology

Received: *27 August 2018*
Accepted: *15 January 2019*
Published: *14 February 2019*

Citation:
Jappe U, Schwager C, Schromm AB, González Roldán N, Stein K, Heine H and Duda KA (2019) Lipophilic Allergens, Different Modes of Allergen-Lipid Interaction and Their Impact on Asthma and Allergy. Front. Immunol. 10:122. doi: 10.3389/fimmu.2019.00122

Molecular allergology research has provided valuable information on the structure and function of single allergenic molecules. There are several allergens in food and inhalant allergen sources that are able to interact with lipid ligands *via* different structural features: hydrophobic pockets, hydrophobic cavities, or specialized domains. For only a few of these allergens information on their associated ligands is already available. Several of the allergens are clinically relevant, so that it is highly probable that the individual structural features with which they interact with lipids have a direct effect on their allergenic potential, and thus on allergy development. There is some evidence for a protective effect of lipids delaying the enzymatic digestion of the peanut (*Arachis hypogaea*) allergen Ara h 8 (hydrophobic pocket), probably allowing this molecule to get to the intestinal immune system intact (sensitization). Oleosins from different food allergen sources are part of lipid storage organelles and potential marker allergens for the severity of the allergic reaction. House dust mite (HDM), is more often associated with allergic asthma than other sources of inhalant allergens. In particular, lipid-associated allergens from *Dermatophagoides pteronyssinus* which are Der p 2, Der p 5, Der p 7, Der p 13, Der p 14, and Der p 21 have been reported to be associated with severe allergic reactions and respiratory symptoms such as asthma. The exact mechanism of interaction of these allergens with lipids still has to be elucidated. Apart from single allergens glycolipids have been shown to directly induce allergic inflammation. Several—in parts conflicting—data exist on the lipid (and allergen) and toll-like receptor interactions. For only few single allergens mechanistic studies were performed on their interaction with the air-liquid interface of the lungs, in particular with the surfactant components SP-A and SP-D. The increasing knowledge on protein-lipid-interaction for lipophilic and hydrophobic food and inhalant allergens on the basis of their particular structure, of their capacity to be integral part of membranes (like the oleosins), and their ability to interact with membranes, surfactant components, and transport lipids (like the lipid transfer proteins) are essential to eventually clarify allergy and asthma development.

Keywords: asthma, food allergy, house dust mite, innate immunity, lipophilic allergens, lipids, peanut, pulmonary surfactants

INTRODUCTION

In recent decades, allergies have become the number one chronic disease in many parts of the world affecting up to 30% of the population in each country (1). Therefore, much effort has been devoted to identifying and characterizing single allergens in order to improve routine diagnostic tests and offer therapeutic interventions. Until lately, however, this work was dedicated to water-soluble allergens because due to their hydrophobic/lipophilic properties lipophilic allergens were not isolated by conventional extraction procedures and were not encountered in IgE screening experiments. The recent discovery of a group of unique lipophilic allergens, termed oleosins, in food allergen sources and their association with severe allergic reactions demonstrated the importance of non-water-soluble allergens and gave impetus to intensify studies on allergen-lipid-interaction and how it impacts the allergic sensitization process (2–4). This review will give an overview of different groups of lipophilic/lipid-associated allergens, the mechanisms of allergen-lipid-interaction known so far and discuss its immunological impact on disease development (**Table 1**). The focus will be on the two most common clinically relevant sources of lipophilic allergens, house dust mite, and peanut.

HOUSE DUST MITE AND PEANUT AS MODEL FOR ALLERGEN-LIPID-INTERACTION

House dust mites (HDM) are the major allergen source found in house dust and a common elicitor of severe respiratory symptoms such as asthma. Surprisingly, more than 80% of individuals suffering from asthma are allergic to HDM (5). This finding triggered an increased interest in the identification of allergens from the two most relevant mite species, the American house dust mite (*Dermatophagoides farinae,* Der f) and the European house dust mite (*Dermatophagoides pteronyssinus,* Der p). Until now, more than 20 allergens have been identified for each species (Der f 1–36; Der p 1–37) (www.allergen.org), some of which with lipophilic properties. Interestingly, the lipophilic allergens have been more often associated with asthmatic diseases (6, 7).

Peanut is an important source of nutritionally valuable lipids (fat content ∼50%). However, it is also one of the most potent allergen sources and a major cause of food-induced anaphylaxis in industrialized countries (8–10). Therefore, peanut allergens have been intensively studied to identify those important for diagnosis and therapy. Among the 16 officially registered peanut allergens (www.allergen.org) two have been identified by us to be associated with lipids, Ara h 8 and Ara h 9 (11, 12). Nevertheless, the accompanying lipids did neither effect their extractability nor their IgE reactivity in immunological test systems, thus their role in peanut allergy did not become apparent at that time. Over the last years the view on lipid-associated allergens has changed, in particular by the discovery of the oleosins, a unique group of water-insoluble membrane proteins. They are absent from diagnostic extracts but have been shown to be potential marker allergens for the severity of the allergic reaction to food (2, 3, 13). These findings have directed our research to the question of the effect of lipids and their association with lipophilic allergens in the context of allergic diseases (see **Figure 1**).

Lipids are small hydrophobic or amphipathic molecules which, according to the International Lipid Classification and Nomenclature Committee, are categorized into 8 groups based on their structural features. These groups are fatty acyls, glycerolipids, sterol lipids, glycerophospholipids, sphingolipids, prenol lipids, saccharolipids, and polyketides (21).

Apart from the lipids interacting directly with allergens, there are also various lipids co-delivered with allergens. They originate either from the allergen source *per se* or from microbes associated to pollen or house dust mites [reviewed by (22)]. Lipids, as part of the allergen source, occur in pollen coats [so called pollenkit, where they exhibit protective functions for the plants (23)], in matrices of plant and animal foods and in animal dander. These lipids can modulate the immune system by interacting with innate lymphocytes, such as NKT cells (24–26). Examples of such immunomodulatory lipids are lipopolysaccharide (LPS) and lipid mediators, i.e., Pollen Associated Lipid Mediators (PALMs).

There is more detailed knowledge on the protein than on the lipid molecules when considering structural interaction or the immunological effect on disease pathomechanisms.

There are several examples in food as well as inhalant allergen sources for structural allergen-lipid-associations, which have in parts already been elucidated, structurally, and/or immunologically. Since they have been already summarized (22, 27), in our review we focus on their potential clinical relevance.

ALLERGENS FROM DIFFERENT SOURCES (FOOD, MAMMALS, ARTHROPODS), AND THEIR ASSOCIATION WITH LIPIDS

In order to understand the effect of structure on the allergenicity of certain allergen sources it is important to understand essential definitions of molecular allergology.

The official nomenclature of single allergens consists of the abbreviated Latin name of the allergen source [the first 3 or 4 letters of the genus, i.e., *Betula* (birch)], the first or the first 2 letters of the species (*verrucosa*) and, in general, the number following the chronological order in which they were identified, (i.e., Bet v 1) (28). Basically, single peanut allergens are named *Arachis hypogaea* (Ara h) 1–17; house dust mite allergens (*Dermatophagoides pteronyssinus* (Der p 1–37), *D. farinae* (Der f 1–36). A huge number of allergens are allocated to only few protein families. Exemplified for peanut, these are the following: the Bet v 1 (*Betula verrucosa*) superfamily, the prolamin superfamily (that includes the lipid-transfer proteins), the conglutin-like storage proteins; 2S-albumins, vicilin-like storage proteins (7S-globulin), the defensins (29), and the oleosins (4). The association with lipids has been described for the allergen families Bet v 1-superfamily, lipid transfer proteins, 2S albumins, 7 and 11S globulins, oleosins, lipocalins, apolipophorins, and the mite allergen groups 2, 5, and 7.

TABLE 1 | Allergens and their interaction with lipids.

Protein family	Source	Allergen	Allergological relevance	Mode of lipid/ligand interaction	Resulting effects
Bet v 1 like	Birch *(Betula verrucosa)*	Bet v 1	Major allergen, associated with mild allergic reactions	Binds ligands *via* hydrophobic pocket	Binds and permeabilizes membranes
	Peanut *(Arachis hypogaea)*	Ara h 8	Minor allergen, associated with mild allergic reactions, marker for pollen-associated food allergy	Binds lipids *via* hydrophobic pocket	Delayed enzymatic digestion (see **Figure 1B**), increased thermal stability, enhanced uptake in intestinal mucosa
Non-specific lipid transfer protein	Peach *(Prunus persica)*	Pru p 3	Pan-allergen, associated with severe allergic reactions (Mediterranean area)	Binds fatty acids in inner hydrophobic cavity	Induction of conformational changes that lead to increased IgE-binding (see **Figure 1C**)
	Peanut *(Arachis hypogaea)*	Ara h 9	Pan-allergen, associated with severe allergic reactions (Mediterranean area)	Potentially binds lipids, phospholipids in inner hydrophobic cavity	Unknown
	Grape *(Vitas vinifera)*	Vit v1	Pan-allergen, associated with severe allergic reactions (Mediterranean area)	binds phosphatidylcholine	Delayed enzymatic digestion
Globulin	Peanut *(Arachis hypogaea)*	Ara h 1	Major allergen, associated with severe allergic reactions	Interaction with phosphatidylglycerol vesicles	Delayed enzymatic digestion
	Mustard *(Sinapis alba)*	Sin a 2	Major allergen, associated with severe allergic reactions	Interaction with phosphatidylglycerol vesicles and mustard lipids	Protection against enzymatic digestion & microsomal degradation, activation of human DCs
2S Albumin	Brazil nut *(Bertholletia excelsa)*	Ber e 1	Major allergen, potentially associated with severe allergic reactions	Lipid-binding hydrophobic cavity is assumed	Co-administration with brazil nut lipids induced IgE and IgG1-response in mice and IL-4 in murine and human CD1d-restricted iNKT cells
	Peanut *(Arachis hypogaea)*	Ara h 2	Major allergen, associated with severe allergic reactions (marker allergen)	None	Might inhibit tryptic degradation of co-administered peanut allergens
Oleosins	Peanut *(Arachis hypogaea)*	Ara h 10 Ara h 11 Ara h 14 Ara h 15	Potential major allergens, associated with severe allergic reactions (potential marker allergens)	Bind phospholipids and lipids *via* hydrophobic domain creating an oil body	Potentially enhanced uptake of oil bodies *via* lipid-carrier-mediated transport mechanism (see **Figure 1E**)
	Sesame *(Sesamum indicum)*	Ses i 4 Ses i 5	Potential major allergens, associated with severe allergic reactions (potential marker allergens)	Bind phospholipids and lipids *via* hydrophobic domain creating an oil body	
	Hazelnut *(Corylus avellana)*	Cor a 12 Cor a 13			
Lipocalin	Cow's milk *(Bos domesticus)*	Bos d 5	Major allergen	Carries hydrophobic molecules, phosphatidylcholine	Insertion into bilayers, protection against enzymatic digestion
	Dog *(Canis familiaris)*	Can f 6	Unknown	Binds LPS	Enhancement of LPS/TLR4-signaling (see **Figure 1D**)
Secreto-globulin	Cat *(Felis domesticus)*	Fel d 1	Major allergen	Potentially binds TLR-ligands	Enhancement of TLR2 and TLR4 signaling
Group 2 mite allergen	House dust mite *(Dermatophagoides pteronyssinus)*	Der p 2	Major allergen, more often recognized by asthmatics	Binds LPS due to structural similarity with MD-2	Enhancement of LPS/TLR4-signaling (see **Figure 1D**) resulting in airway Th2 inflammation
	House dust mite *(Dermatophagoides farinae)*	Der f 2	Major allergen, more often recognized by asthmatics	Binds LPS due to structural similarity with MD-2	

(Continued)

TABLE 1 | Continued

Protein family	Source	Allergen	Allergological relevance	Mode of lipid/ligand interaction	Resulting effects
Group 5/7 mite allergen	House dust mite *(Dermatophagoides pteronyssinus)*	Der p5	Minor allergen, more often recognized by asthmatics	Hydrophobic cavities that might bind apolar ligands	Potential stimulation of TLR2 (see **Figure 1D**)
		Der p 7	Minor allergen, more often recognized by asthmatics	Hydrophobic cavities that might bind apolar ligands	
Group 13 mite allergen	House dust mite *(Dermatophagoides pteronyssinus)*	Der p 13	Minor allergen	Selective binding of fatty acids in inner cavity	Induction of airway epithelial cell activation through TLR2-MyD88-NF-κB and MAPK-dependent mechanisms (see **Figure 1D**)
Group 14 mite allergen	House dust mite *(Dermatophagoides pteronyssinus)*	Der p 14	Minor allergen	Potential transporter of lipids	Unknown
Group 21 mite allergen	House dust mite *(Dermatophagoides pteronyssinus)*	Der p 21	Minor allergen	Potentially binds lipids from house dust mite	Activation of airway epithelial cells through TLR2 signaling

Major allergen: recognized by >50% of individuals allergic to the culprit allergen source. Minor allergen: recognized by <50% of individuals allergic to the culprit allergen source.

The respective proteins possess hydrophobic / lipophilic properties which provide the prerequisites for allergen-lipid-interactions either *via* binding through hydrophobic cavities (15, 30–32), ionic (33), or hydrophobic bonds (34).

These intrinsic properties of the allergens most probably have an impact on their allergenicity. Basically, allergens can carry lipids ("lipid cargo") (27), and these lipids can alter the allergenicity of allergens by modifying their structure and biochemical properties. On the other hand, it is most plausible that lipids are carriers for allergens (see oil bodies-oleosins). The structural prerequisites are different and only partly elucidated. Some lipids change the tertiary structure of proteins so that allergenic epitopes are exposed to IgE antibodies (see paragraph heading Lipid Transfer Proteins) (see **Figures 1A–D**).

FOOD ALLERGEN SOURCES

Bet v 1 and Its Homologs in Food Allergen Sources

It was shown that the Bet v 1-molecule has a hydrophobic pocket binding various physiologically important lipophilic ligands, including free fatty acids (30, 35, 36). We could show that the Bet v 1-homolog of peanut and relevant marker allergen for pollen-associated food (class II) allergy, Ara h 8, purified from roasted peanuts, possesses a hydrophobic pocket where lipids are attached to the protein (12). This is noteworthy as there is some evidence for a protective effect of lipids delaying the enzymatic digestion and supporting the uptake of allergens by intestinal mucosa cells (see **Figure 1B**) (12, 22).

Lipid Transfer Proteins (LTP)

Nonspecific lipid transfer proteins (nsLTP) are a class of proteins with potent allergenic representatives in pollen and food allergen sources. Basically, characteristic features of LTPs are a low molecular weight of ∼10 kDa, and a hydrophobic cavity which pervades the molecule and allows the hosting of ligands such as fatty acids. In the Mediterranean area LTP-sensitization is associated with severe allergic reactions (11). However, some single cases occur in Northern Europe as well (37), and their number is increasing. Peanut possesses three lipid transfer proteins. The first one is Ara h 9 (with two isoforms) (11), to which 38.5% of peanut-allergic individuals in the Mediterranean area were sensitized, whereas in the same population IgE-reactivity to the storage proteins and major allergens Ara 1, Ara h 2, and Ara h 3 was altogether 4.8%. Further, there are Ara h 16 and Ara h 17, for which the WHO/IUIS[1] allergen nomenclature documentation exists but no publications (www.allergen.org). For Ara h 9, Ara h 16, and Ara h 17 the lipid-association has not yet been elucidated in contrast to other members of this protein family. However, Krause et al. had evidence that the peach allergen *Prunus persica* 3 (Pru p 3) or Ara h 9 may be the primary sensitizing allergens in those cases where peanut storage proteins or Ara h 8 were not responsible for primary sensitization to peanuts. That makes Ara h 9 an important addition to the component-resolved diagnosis. Furthermore, a deeper insight into its sensitization route could provide important information to develop methods for the prevention of food allergy (11). The 3-dimensional structure is very similar among non-specific (ns) LTPs due to characteristic disulfide bonds (11). Sequence identity among LTPs is generally low with the exception of Pru p 3 and *Malus domesticus* (Mal d) 3 (apple). However, nsLTPs from various allergen sources may differ considerably with regard to their potential allergen cross-reactivity.

A detailed review on LTPs was published in the special issue of Frontiers in Immunology, Role of Lipids in the Dynamics of Allergic Airway Inflammation by Scheurer and Schülke (38). We chose Pru p 3 to include an exemplified report on

[1]Available online at: www.allergen.org Official Homepage of the WHO/IUIS Allergen Nomenclature.

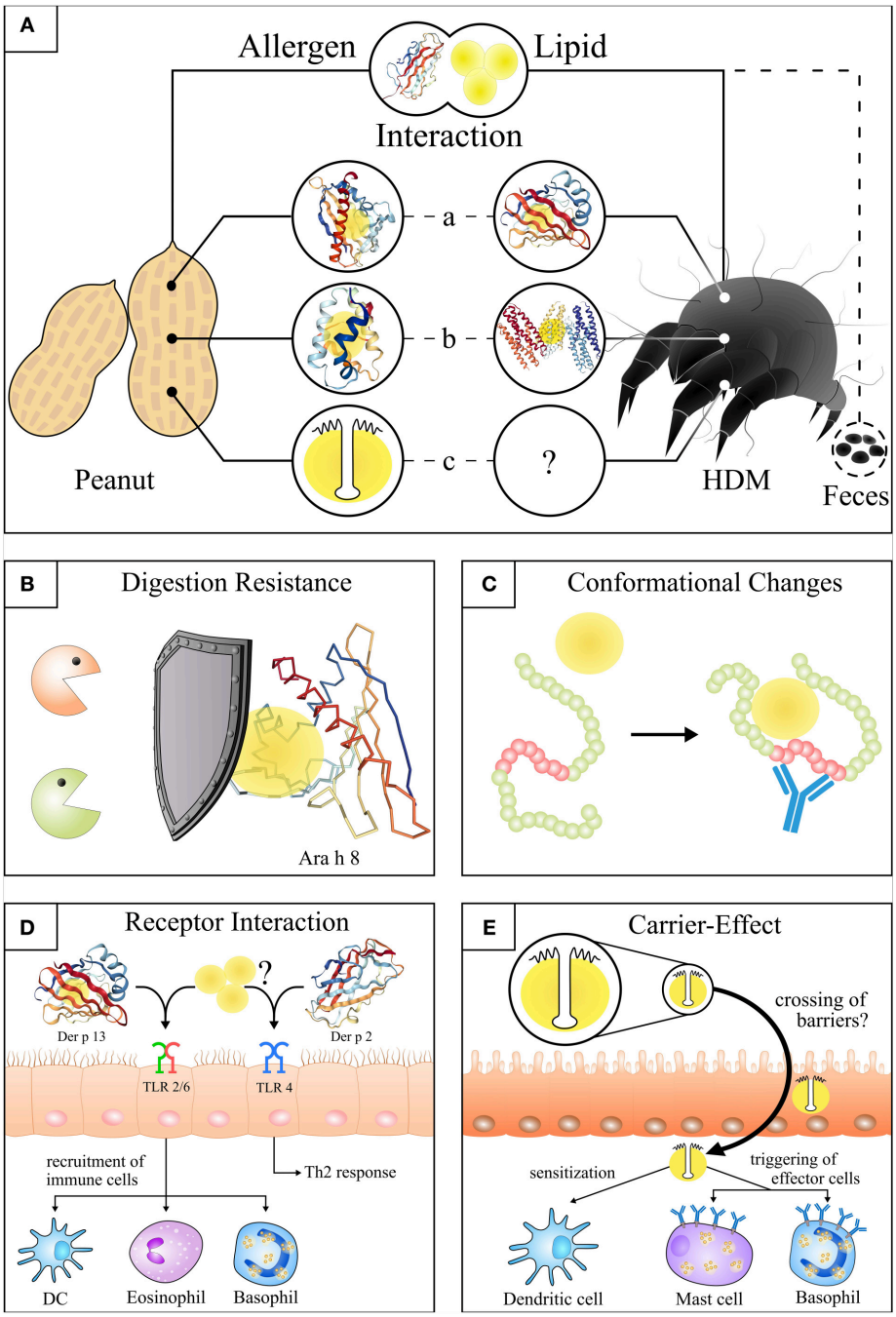


FIGURE 1 | Structures and effects of the allergen-lipid-interaction in peanut and house dust mite. **(A)** Illustration of the molecular interaction of lipids and allergens from peanut and house dust mite. (a) Lipids bound to the hydrophobic pocket of Ara h 8* (14) (PDB ID: 4M9B) and Der p 13**. (b) Lipids integrated into the hydrophobic cavity of Ara h 9*** and Der p 5 (15) (PDB ID: 4M9B). (c) Lipids attached to the hydrophobic domain of peanut oleosins****. **(B)** Attached lipids delay or prevent the digestion of lipophilic proteins (simplified cartoon: lipids bound to Ara h 8). **(C)** Lipid binding induces conformational changes of allergens that lead to the exposure of hidden epitopes (red part). **(D)** Potential initiation of HDM sensitization through activation of TLR2 by Der p 13** and associated or free lipids (left side) or Der p 2 (PDB ID: 1KTJ) mediated TLR4 activation in bronchial epithelial cells (right side). **(E)** Uptake of oil bodies**** (and intrinsic proteins e.g. oleosins) by a lipid carrier-mediated transport in the gut as a potential route of sensitization. The individual structures of proteins were visualized by use of NGL viewer (16). *Lipids were integrated according to the analogous protein Bet v 1 (17) (PDB ID: 4A83). **Protein structure of the Der p 13 homologues Der f 13 (18) (PDB ID: 2a0a) is shown as there is no PDB structure of Der p 13 available. ***For visualization of the non-specific lipid transfer protein (nsLTP, Ara h 9) the structure of the similar nsLTP from maize (19) (PDB ID: 1MZM) was used. ****Simplification of a peanut oil body. A more realistic structure can be looked up at Jappe and Schwager (20).

LTPs in the context of our own review. Pru p 3 has been investigated in detail for its lipid-association, is considered the clinically most important and best characterized food LTP and a marker allergen for LTP-sensitization (39). For peach and hazelnut LTPs investigations with and without lipids have been performed, suggesting that the binding of lipophilic ligands altered the cavity ("structural plasticity") (40). Dubiela et al. have reported the binding of the following substances to Pru p 3: lauric acid, cis-parinaric acid, palmitic acid, and linoleic acid. The authors investigated whether peach-LTP-ligand (lipid) interaction affected IgE-binding in sera from peach allergic patients. It was shown that the region most probably affected by the structural plasticity and the conformational changes of the hydrophobic cavity induced by oleic acid binding contained the major IgE epitope responsible for severe reactions. Interestingly, this fatty acid also bound recombinant Pru p 3 which is in contrast to our own observations for the Bet v 1-homolog in peanut, the Ara h 8 (12). However, it was shown that the preincubation of recombinant (r) Pru p 3 with oleic acid increased the IgE-reactivity of the sera of 10 peach allergic patients in an ELISA when compared with rPru p 3 alone. The next step was a cellular diagnostic test, the basophil activation test, which confirmed the ELISA results: rPru p 3 plus oleic acid increased the number of activated basophils when compared with rPru p 3 alone (40).

Similar results were obtained with a grape *Vitas vinifera* nsLTP (Vit v 1) in the presence of phosphatidylcholine which was investigated in an *in vitro* assay where digestion-protected LTP increased the release of histamine from basophils (41).

The knowledge about the oleic acid effect regarding increase of allergenicity can be used (A) in the conception of a safer immunotherapy by reduction of side effects; (B) the increase in IgE-binding has the potential of improving the sensitivity of component-resolved diagnostic tests (40).

2S Albumins and 7S/11S Globulins

Ara h 1, a storage protein (7 S globulin) of peanut and a major allergen as well as Sin a 2, an 11 S globulin in mustard (*Sinapis alba*) belong to the cupin superfamily. Both food allergen sources induce severe reactions. Sin a 2 has already achieved the status of a marker allergen for the severity of the reaction (42) and is responsible for cross-reactivity between mustard, peanut and tree nuts. In 2016, Angelina et al. could demonstrate one way of interaction between the globulins and lipids and the respective effects (43). In general, allergens are processed by dendritic cells (DCs) *via* endolysosomal compartments, enabling their presentation on MHC II-peptide complexes to T cells. Angelina et al. showed that mustard lipids and phosphatidylglycerol vesicles associate with Sin a 2 and diminished its uptake by DCs (43). In the same study they showed that the presence of mustard lipids together with Sin a 2 shifts the cytokine profile of DCs in a more Th2-favored direction and enhances IL-β release compared to Sin a 2 stimulation only. Further, the authors showed that in THP-1 cells mustard lipids and phosphatidylglycerol vesicles, but not peanut lipids inhibited NF-kB/AP-1 activation induced by a TLR2 ligand. Altogether, this supports the potency of these molecules to contribute to the allergic sensitization process. Further, in the same study, there is evidence that lipids also influence the direct allergen-specific activation of effector cells. The presence of mustard lipids and phosphatidylglycerol vesicles protected Sin a 2 from gastric digestion and preserved it's IgE-binding property. Phosphatidylglycerol vesicles also protected Ara h 1 from gastric digestion to a considerable extent, and 40% of the IgE-binding capacity was retained. However, Ara h 1 was not protected from intestinal digestion, but the fragment still had IgE-binding capacity for some time.

Ber e 1, a seeds storage 2S albumin in Brazil nut (*Bertholletia excelsa*), has a compact alpha-helical, disulphide-bridged rigid structure. It is hypothesized that there is a lipid-binding hydrophobic cavity, and that the allergenicity of Ber e 1 in mice depends on the presence of lipids from the Brazil nut matrix (44) as this combination stimulated murine and human CD1d-restricted iNKT cells that produced IL-4 but not IFN-γ.

The results described here make it plausible that clinically relevant single allergens pass the gastrointestinal tract in a structural condition that is still immune competent (43).

Although, Ara h 2 is a 2S albumin from peanut and shows some homology to the LTPs (both belong to the prolamin superfamily), the domain responsible for lipid-binding is not present in the Ara h 2 molecule (45). However, Ara h 2 functions as a trypsin inhibitor and is able to prevent the degradation of further accompanying allergens such as Ara h 1 (46). In addition, roasting of peanut increases the IgE-binding potential of Ara h 2 by up to 90-fold (47). This issue is thought to be attributed to the Maillard reaction which occurs during heat treatment und may lead to the creation of "neo-epitopes" on allergens (48, 49). Here, the presence of lipids, especially triacylglycerols with unsaturated fatty acids, might unintentionally lead to the creation of Maillard reaction products (MRP) as peroxidation of fatty acids generates α-dicarbonyls, highly reactive MRP precursors (50). The phenomenon of an enhanced IgE-binding after roasting is not restricted to Ara h 2 but has also been observed for other peanut allergens such as Ara h 1, Ara h 8, Ara h 12, and Ara h 13 (defensins) and Ara h 10, Ara h 11, Ara h 14, and Ara h 15 (oleosins) (12, 13, 29, 51).

Oleosins

Oleosins are unique lipophilic allergens (20) that can be found in oil-rich seeds and plant pollen but also in mosses, ferns and algae (52, 53). They are an integral part of the phospholipid layer of oil bodies, the lipid reservoir of plants (53). Oleosins have been overlooked in their capacity of being allergens for many years due to their poor extractability and the many methodological problems that had to be faced regarding their isolation and preparation. Although some peanut allergens are known to be associated with lipids they are—in contrast to oleosins—soluble in aqueous solutions. The main characteristic of oleosins is their relatively large conserved hydrophobic domain (∼7 kDa) which is anchored in a lipid storage organelle termed oil body, lipid body or oleosome. The amphipathic N-terminal and C-terminal domains of the oleosins which flank the hydrophobic domain are most often smaller and reside on the surface of the oil body to prevent their coalescence by steric hindrance and electrostatic repulsion [for a schematic model see (20)]. Based on their

structural features and their tight association with lipids, oleosins are missing in aqueous-based diagnostic extracts and are yet not available as single allergens for routine diagnostic allergy tests. However, their potential to identify patients suffering from severe allergic reactions has been demonstrated in sesame allergy (2), hazelnut allergy (3), and peanut allergy (13). So far, only Schwager and co-authors managed to use oleosins purified from roasted and raw peanuts for further immunological investigations such as the basophil activation test. However, up to now it is not clear whether a potential aggregation of oleosins in an aqueous environment enhances their allergenicity through the exposure of multiple IgE-binding epitopes located in the amphipathic domains that might crosslink the Fcε receptor on basophils more efficiently.

The past years of research have raised the question whether the sensitization mechanism used by oleosins is different from that of other lipid-associated allergens. This question cannot be fully answered by the literature at present but several studies indicate that a lipid carrier-mediated mechanism is involved in the transfer across epithelial barriers (see **Figure 1E**) (54). Experimental data in mice suggest a more rapid uptake of substances entrapped in artificial oil bodies *via* gut and skin (55, 56). These observations might be important in many ways. First of all, oil bodies have been reported to be associated with proteins/allergens other than oleosins, and thus might act as a transfer vehicle that facilitates the contact of extrinsic allergens with immune cells (57, 58). Secondly, it has been shown that oleosins can be present even in refined oils which are typically used as ingredients in ointments and skin care products of patients with atopic eczema (59, 60). This raises the question whether the treatment of eczema patients with the respective products may put these patients at risk of being sensitized.

Palladino et al. recently published the potential "adjuvanticity" of peanut lipids with skin keratinocytes as effector cells (61). They investigated human primary keratinocytes, exposing them to all major peanut lipid classes together with or in the absence of peanut storage proteins and major allergens, Ara h 1 and Ara h 2. The peanut lipids were obtained from roasted peanuts. The group demonstrated a direct effect of peanut lipids on human keratinocytes triggering an inflammatory mediator production, because keratinocytes were able to recognize peanut lipids as exogenous stimulus. Since the pro-inflammatory mediators were elsewhere described as inducers of a barrier disruption which allows allergen penetration and subsequent allergic inflammation, the authors hypothesize a potential role of peanut lipids as adjuvant for peanut allergens (61).

MAMMALIAN ALLERGENS

Lipocalins

Members of this protein family transport small hydrophobic molecules like some lipids, steroid hormones, retinoids, pheromones, fragrances, and bilins. Lipocalins are molecules of a molecular weight between 16 and 22 kDa with different primary but similar tertiary structures. They have a binding pocket for extrinsic molecules, however, their precise role as allergens in the allergenic pathomechanism is still obscure. They are ubiquitous and can be found in arthropods, plants, and bacteria as well as mammals (62). Mammalian lipocalin allergens are carried by dander, saliva and urine. Allergens of this group consist of almost all significant inhalant mammalian allergens (mammalian dander allergens) and are distantly related to cytoplasmic fatty acid binding proteins. Allergenic representatives are *Equus caballus* (Equ c) 1 (horse), *Canis familiaris* (Can f) 1, Can f 2, Can f 4, and Can f 6 (dog), *Felis domesticus* (Fel d) 4 and Fel d 7 (cat), and *Bos domesticus* (Bos d) 2 (cattle). The dog lipocalin Can f 6 revealed considerable cross-reactivity between dog, horse, and cat. Further lipocalins have been identified in guinea pig, hamster, mouse, rat, and rabbit (63).

As such, lipocalins are not highly allergenic which may be due to the fact that most of their representatives show an amino acid identity of between 40 and 60% with endogenous human proteins which inhibits a proper immune recognition. The resemblance to the "immunological self" is thought of as one of the main reasons of low grade allergenicity (64) as they are not optimally recognized by human T cells which may favor the raise of Th2 responses.

Dog lipocalins bind LPS, thereby enhancing LPS/TLR4-signaling in for example primary macrophage-like cells (65).

β-lactoglobulin from milk, Bos d 5, is a major cows' milk allergen and also belongs to the lipocalin superfamily. It is known to carry hydrophobic molecules (62). Bos d 5 inserts into lipid bilayers and its interaction with phosphatidylcholine protects it from digestion in an *in vitro* gastroduodenal setting [reviewed by (22)].

ARTHROPOD ALLERGENS

Mite Protein Groups 2, 5, and 7

The HDM allergens Der p/f 2, Der p/f 5, Der p/f 7, and Der p/f 21 have lipophilic properties and are able to stimulate the immune system *via* mimicry of receptor ligands as shown in mouse models (15, 32, 66–68). Group 2 mite allergens are able to bind LPS because of their structural similarity to MD-2 (31). MD-2 is the component of the TLR4 complex that is responsible for the LPS-binding (69). Der p 2 drives TLR4 signaling followed by Th2 inflammation of the airways in wild-type mice but not TLR4-deficient mice. In this, Der p 2 acts as an autoadjuvans in the sensitization process (67). (For additional details see section heading **Lipids (and Allergens) and TLR-interaction**).

Der p 5 dimer and Der p 7 structures have hydrophobic cavities that may bind apolar ligands (15, 32). Therefore, it seems possible or even likely that this lipid-cargo-situation is synergistic between these allergens from one source in the stimulation of TLR2 (70). Der p 7 was described to bind polymyxin B, which is a bacterial lipopeptide that can bind and neutralize LPS. The crystal structure of Der p 7 reveals homology to the human lipid-binding protein family including LBP and bactericidal permeability-increasing protein (BPI) (32). This is interesting in so far as house dust mites are transporters of bacteria. However, lipid-binding studies failed to show interaction of Der p 7 to LPS or to distearoyl phosphatidyl choline (DSPC). A natural ligand for Der p 7 has not been identified yet (32).

Cytoplasmic Fatty Acid Binding Proteins (FABPs)

Mite Protein Group 13

The FABP family consists of small proteins that are usually not secreted but remain in the cell cytoplasm. They are involved in the binding and transport of fatty acids in their inner cavity in vertebrates and invertebrates (71). Phylogenetic analyses revealed a potential emergence of ancestral FABP genes from lipocalins (72, 73). FABPs have been identified as allergens in diverse mites (group 13 allergens). Der p/f 13 has sequence similarities with FABPs and may reveal innate immune signaling properties *via* interaction with mite or microbial lipids (74). In their study, Satitsuksanoa et al. showed that Der p 13 is able to transport certain lipids (fatty acids) and that the proteins' binding to hydrophobic ligands is selective (70).

Nonetheless, their IgE-binding frequency is considered to be very low (70). This might be explained by the fact that FABPs reside inside the mite bodies and are not secreted as feces which can be more easily inhaled (75).

Nevertheless, it was shown that Der p 13 induced airway epithelial cell activation through TLR2-MyD88-NF-kappaB and MAPK-dependent mechanisms (see **Figure 1D**), however, the structural integrity of Der p 13 was not required suggesting an effect of the protein' s lipid cargo (70). Up to now, only the tertiary structure of Der f 13—and not of Der p 13—was solved using nuclear magnetic resonance (see **Figure 1A**).

Apolipophorin

Mite Protein Group 14

Lipophorins are lipoproteins found in the hemolymph of most insects. Depending on the insect species they contain several apoproteins (76). Lipophorin is considered to be part of lipid bodies and transport particles of the hemolymph. Apolipophorins are poorly soluble in aqueous extracts.

Der p 14 is an LTP that bears an apolipophorin-like sequence in its N-terminal domain which shows certain similarity to the human apolipoprotein B100 and insect apolipophorins. Similar to other proteins of the apolipoprotein family, the biological function of Der p 14 seems to be the transport of lipids as it was mainly found in lipid bodies and lipid transport particles of the haemolymph in house dust mites (77). In solution, members of the group 14 HDM allergens have been found to be degraded into smaller fragments by proteolytic enzymes derived from the mite itself (e.g., group 1 allergens). However, the resulting peptides seem to be more potent in IgE-binding compared to the intact allergen and are able to trigger immune cells of allergic patients to release IL-4 and IL-13 (77, 78).

GLYCOLIPIDS: A SPECTRUM OF FUNCTION BETWEEN ANTIGEN AND ADJUVANTS

Although lipids are not—like proteins—the primary target of the players of adaptive immune reactions, there is some evidence that they play a specific role in the pathomechanism of allergy by interacting with innate lymphocytes. Here we focus on the example of lipids bearing the sugar component, so called glycolipids being recognized by invariant Natural Killer T cells (iNKT).

Furthermore, since to date, there are no described glycolipids neither from peanut nor HDM, we discuss the role of iNKT cells in food allergy, based on the few available examples.

In food allergy, the immune response is evidently biased toward a phenotype dominated by Th2 cytokines. Why some food proteins induce this type of response is still a matter of debate. However, the induction of an allergic phenotype requires a primary source of IL-4. Together with basophils, iNKT cells have been identified to be the cells providing the IL-4 that determines Th2 cell differentiation and IgE production (79) and were implicated in the induction on several allergic diseases including asthma (80, 81).

In contrast to conventional protein-reactive T cells, iNKT cells recognize glycolipids as antigens presented on the MHC class-I-like molecules of the CD1 family. Asperamide B, a glycosphingolipid isolated from the ubiquitous fungus *Aspergillus fumigatus*, is the only example of a fully chemically-defined glycolipid able to induce directly allergic inflammation (independently from allergen), that resulted in airway hyperreactivity (82).

This fundamental difference and the fact that allergen sources in general contain glycolipids that can activate iNKT cells has some implications. For example, in the case of food allergy, a food-related glycolipid inducing IL-4 production by iNKT cells can act as an adjuvant able to favor the Th2 cell differentiation. In addition, while protein food allergen exposure may activate allergen-specific T cells or mast cells, the responses to exogenous (and perhaps food-related) glycolipids by iNKT cells allows the induction of allergic inflammation independently of Th2-type allergen sensitization. Moreover, the simultaneous activation of Th2 cells and iNKT cells may result in the amplification of the allergic reaction.

In a mouse model of nut allergy, the lipid-binding major allergen Ber e 1 induced specific IgE- and IgG1-antibodies only when applied together with neutral or common phospholipids derived from Brazil nuts. Antibody production was lower in NKT-deficient mice, suggesting the involvement of iNKT cells to be essential for the adjuvant activity of nut lipids (44). A recent study addressing the sensitization to one of the more frequent plant food allergens in southern Europe, Pru p 3, suggested that a lipid-ligand isolated from peach peel could act as an adjuvant for Pru p 3 *in vivo*, since the co-administration of allergen and lipid-ligand induced higher levels of IgE than allergen alone, and this effect seemed to be mediated by CD1d. In addition, the lipid-ligand was recognized by an iNKT cell line (83). Milk allergy also has a link to iNKT cell activation. Children with milk allergy had a reduced number of iNKT cells in peripheral blood in comparison to children suffering from food allergy (but not milk allergy) and healthy children. Interestingly, the remaining iNKT cells had a strong bias toward IL-4 and IL-13 production in contrast to non-allergic individuals. Further on, the glycolipid sphingomyelin present in cows' milk was identified as the responsible agent for the expansion and the IL-4

production by iNKT cells in children with milk allergy (84). A later study by the same laboratory showed that children who received oral immunotherapy (OIT) were shown to experience a reconstitution of the iNKT numbers and their switch from a Th2 toward a Th1 phenotype (85). Using a murine model, Rajavelu et al. reported that iNKT cells are required for the development of eosinophilic esophagitis driven by allergy to peanut or corn, as iNKT-deficient mice were protected. And either intravenously or intranasal administration of a potent iNKT cell agonist alone, was able to induce experimental eosinophilic esophagitis (86).

The few available studies strongly suggest that iNKT cells and glycolipids present in allergen sources play a prominent role in the pathogenesis of food allergy. This is an attractive target for food allergy therapies since interrupting the activation or effector function of iNKT cells may be highly effective in the treatment of multiple forms of food allergy, especially those where the conventional treatments are insufficient.

As stated above, glycolipids that are presented on CD1 molecules and recognized by the iNKT cell TCR have a function as antigens, but there are other lipid classes that can also activate iNKT cells while functioning as adjuvants upon stimulation of Toll-like receptors (TLRs) on antigen-presenting cells, such as DCs. Lipid-recognition through this pathway modifies the production of cytokines and provokes changes in the expression pattern and dynamics of CD1 molecules, hence influencing the presentation of glycolipids as antigens. In nature, lipids normally form mixed aggregates (micelles) when they come in contact with aqueous biological solutions, which is also the base for the formation of membranes and lipid-protein complexes. Thus, as is the case for the most sources of allergens, complex biological particles may contain glycolipid antigens for iNKT cells, physically associated with protein-allergens as "cargo" (27) and in addition to other TLR lipid-agonists (i.e., lipopeptides, lipopolysaccharide) (87). The encounter by DCs of such "lipid-allergen package" at mucosal surfaces, may deliver signals that differ in time and significance for allergic responses. For example, the immediate and purely innate signal mediated by the recognition of TLR lipid-agonists would provoke the expression of co-stimulatory molecules and the release of IL-12/IL-18, while also causing the upregulation of CD1 molecules for glycolipid presentation (88, 89). In turn, activation of iNKT cells by glycolipid antigens and/or IL-12/IL-18 would amplify the cytokine response necessary for the polarization of $CD4^+$-T cells (90, 91) and the further activation of the DCs (92), providing in this way a bridge between the innate and adaptive immune response. Finally, priming *via* TLRs and cell-to-cell interaction with iNKT cells results in the maturation of DCs with an increased capacity for protein-allergen presentation on MHC class-II to conventional $CD4^+$ -T cells for the purely adaptive response part of the allergic reaction. A more detailed insight of the molecular pathways involved in the lipid-TLR-iNKT axis could provide the base for additional lipid antigen-specific T cell modulation in allergic responses.

LIPIDS (AND ALLERGENS) AND TLR-INTERACTION

The TLR2/TLR6 and TLR2/TLR1 heterodimers as well as TLR4 are the only known TLRs that recognize bacteria-derived lipids. These ligands are lipoproteins (93, 94) and lipopolysaccharides (95), respectively, and are naturally occurring contaminations of allergen sources like house dust mite and food. The receptors are associated with allergy and asthma, but data regarding their exact role are conflicting. There are several reviews summarizing association studies that link single nucleotide polymorphisms in TLR4 or TLR2 with a reduced risk for asthma and other allergic disorders, while other studies were not able to prove such a correlation (96–98). Further, *in vivo* mouse models showed that TLR2 and TLR4 ligands can lead to either protection or worsening of allergic symptoms, which is in strict dependency of the ligand concentration and time of administration (96, 99–101). Thus, the induction of NF-κB activation and the release of proinflammatory cytokines before sensitization may lead to an induction of regulatory responses and a shift from Th2 to Th1 responses, whereas the same cytokines in an already sensitized and allergic host can lead to an increase of the allergic symptoms.

Lipid recognition through both, TLR2 heterodimers and TLR4, differs concerning the mechanism of ligand recognition and the induced signaling cascades. In order to initiate signaling, TLR2 (93, 94, 102) needs to heterodimerize with either TLR1 or TLR6 (103, 104). The determination of which heterodimer is engaged is driven by the lipid part of the lipoproteins: in general, diacylated lipoproteins signal through TLR2/TLR6 (105) and triacylated lipoproteins signal through TLR2/TLR1 (106). Despite the differential requirements of TLR1 or TLR6 and the subsequent formation of distinct TLR2 heterodimers, the induced signaling of different lipopeptides is rather similar and dependent on MyD88 (107).

In lipopolysaccharides, the lipid part (i.e., lipid A) of the molecule is the component that drives the innate immune reaction (108). A prototypical lipid A molecule (e.g., from *Escherichia coli*) contains six fatty acids, two of which are secondary fatty acids on the first hexosamine. The binding of lipid A to MD-2 leads to a conformational change in MD-2 and the subsequent binding to TLR4 (109, 110). Interestingly, the number of acyl chains also causes TLR4-dependent species-specific differences in the immune responses toward lipid A: the 4-fold acylated precursor of lipid A is completely inactive and can be used as an antagonist in human cells, but is comparably active in mouse cells (111). The TLR4-mediated signaling is the most complex of all TLRs since both central adaptor molecules, MyD88 as well as TRIF, are involved (112–114).

Apart from the signaling pathway induced by the lipid ligands itself, some allergens can modulate TLR responses by their lipid-binding character. Herre et al. for example showed that Fel d 1 (an uteroglobin) and Can f 6 (a lipocalin) enhance ligand-induced TLR2 and TLR4 signaling, although in contrast to Der p 2, it is not by mimicking the biological function of MD-2. The authors hypothesize that both allergens can directly bind lipid ligands of TLRs and enable a more efficient transfer to cell surfaces (65).

Further, when respiratory epithelial cells were stimulated with Der p 13, presumably embedding fatty acids, the production of IL-8 and GM-CSF was triggered in an TLR2-, MyD88-, MAPK-, and NF-κB- dependent signaling pathway (70).

LTP IN LIPID-MEDIATED INFLAMMATION

There are not only plant-derived LTP that were shown to have considerable relevance for allergic inflammation. Several members of human LTP have a peculiar function in the immune recognition of lipids and can either enhance or reduce the immune response in the context of TLR activation. In particular the human LPS-binding protein (LBP) has been assigned a role in the development of allergic asthma in this context (115–117). LBP was found to be expressed not only in the liver, but also in the lung and the intestine (118–120), supporting organ specific activity of LBP in regulating immune responses. Although not established so far, interaction of human LTPs with hydrophobic allergens or with lipids brought as cargo of lipid-binding allergens has to be considered and is likely to shape inflammation and with this also the allergic immune response in these organs. Insights into the function of lipid-binding allergens and allergen-lipid transfer proteins in this context are rather limited so far, but are worth addressing for a better understanding of allergic inflammation. Mechanistic studies might be inspired by the knowledge of human LTPs, comprising besides LPS-binding protein (LBP): the phospholipid transfer protein (PLTP), cholesteryl ester transfer protein (CETP), bactericidal permeability-increasing protein (BPI), and the palate, lung, and nasal epithelium clone proteins (PLUNC, also called BPI-fold containing proteins, BPIF) (121, 122). Whereas, PLTP and CETP are involved in the management of endogenous lipids, the major function of BPI is in the antimicrobial defense of pathogens by binding of bacterial lipopolysaccharide (LPS) and direct antimicrobial activity (123), whereas the acute-phase protein LBP confers LPS-transport to cellular LPS receptors TLR2 and TLR4 and thereby sensitizes the immune response to minute amounts of LPS (124). All members of this family have a common hydrophobic cavity and are combined by structural homology into the tubular lipid-binding (TULIP) domain superfamily, also including the house dust mite allergens Der p 7 and Der f 7 (125, 126).

For the human CETP a tunnel mechanism was determined shuffling molecules of cholesteryl ester through a continuous tunnel formed by two interconnected hydrophobic pockets (127). LBP binds the microbial lipid LPS, and di- and triacylated lipopeptides conferring a transport to cell surface cluster of differentiation CD14 and subsequent activation of the cellular receptors TLR4/MD-2 or TLR2 in cooperation with TLR1 or TLR6 (128–130). Studies in LBP-deficient mice strongly support a role of LBP in the pulmonary immune response to infections (131, 132). The crystal structures of BPI (133) and LBP (134) revealed bound phospholipids in hydrophobic pockets distinct from the LPS binding sites of both proteins. For LBP, functional interaction with phospholipids has been shown in the context of phospholipid transport (135, 136). Moreover, phospholipid binding is of particular interest for the regulation of LBP immune functions. Inhibitory activity on LPS-mediated cell activation is reported for anionic phospholipids (137), surfactant lipids (138–140) and oxidized 1-palmitoyl-2-arachidonoyl-phosphatidylcholine (oxPAPC) (141, 142). The strong capacity of anionic lipids to reduce LBP-induced inflammation could be relevant in the case of plant allergens carrying phosphatidylglycerol lipids that are common in plant membranes (143). Recently, we demonstrated that LBP also interacts with the phospholipid membrane of immune cells. LBP is abundant on the surface of blood derived monocytes of healthy donors, and cell-associated LBP was shown to mediate a transport of LPS to intracellular compartments. Co-localization and functional studies support an involvement of LBP-mediated transport of LPS to intracellular caspases and triggering of inflammasome activation (144). These data demonstrate that phospholipid binding can interfere with the LPS-transport function of LBP, inhibit activation of TLR4 or feed LPS to intracellular receptors.

PLUNC (palate, lung and nasal epithelial clones) proteins are expressed in all epithelial surfaces of the nasopharynx, the upper airways, and the lungs (122). Besides indication for a surfactant function (145), a role of PLUNC proteins in nasal and pulmonary immune control is supported by a number of publications showing involvement in chronic rhinosinusitis (146), in the defense against *Klebsiella pneumonia* and *Pseudomonas aeruginosa* (147, 148). Expression of PLUNC proteins is upregulated by viral and bacterial infection. However, the mode of action of PLUNC proteins is not yet well-understood. The identification of lipid ligands of PLUNC proteins is ongoing research. *In vitro* binding assays showed LPS binding for some PLUNC isoforms isolated from nasal fluids (149). High affinity binding of PLUNC 1 to DPPC (dipalmitoylphosphatidylcholine) has been reported, suggesting that the major phospholipid component of pulmonary surfactant might be an endogenous ligand (150). Latherin, an allergen from horse sweat and saliva is a member of the PLUNC protein family and an inducer of IgE antibodies in horse-allergic subjects (151). The small soluble protein has an unusual surfactant activity reducing the surface tension of water that depends on the unfolding of the globular structure resulting in exposure of hydrophobic protein residues at the air-water interface (152).

SURFACTANT INTERACTION OF (AERO)ALLERGENS

Inhaled allergens and allergen-associated lipids will eventually come in contact with the air-liquid interface of the lungs (153). The interface is formed by pulmonary surfactant which is produced and secreted by type-II bronchial epithelial cells and covers the epithelium of the airway mucosa as a lipid monolayer. Surfactant consists of a complex mixture of ∼90% lipids and ∼10% proteins. Phospholipids make about 80–85% of the surfactant lipid phase with the major phospholipid component dipalmitoylphosphatidylcholine (DPPC) that accounts for about 50% of the lipid mass in surfactant. Minor surfactant components

are phosphatidylglycerol (PG) with 7–15% of the lipid mass and small quantities of phosphatidylinositol (PI), phosphatidylserine (PS) and phosphatidylethanolamine (PE). The hydrophobic surfactant proteins (SP), SP-B and SP-C, are closely associated with the phospholipids in the surfactant film, SP-B being essentially involved in stabilizing the surfactant structure and function by creating phospholipid lamellar bodies associated with the monolayer (154). SP-B deficiency, therefore, is lethal (155). SP-C also has a stabilizing function for the surfactant film. In *Aspergillus fumigatus*-induced allergic airway inflammation the amounts of SP-B and SP-C were both found to be strongly reduced by 50% in brochoalveolar lavage fluid (156).

The soluble surfactant proteins SP-A and SP-D are both centrally involved in the pulmonary immune response, defense to pathogens and the regulation of cellular immune functions of the alveolar macrophage (157–159). SP-A and SP-D belong to the family of collectins. The typical carbohydrate-recognition domain of these lectins preferentially recognizes carbohydrates exposed on the surface of pathogens. Recently, a role for SP-A as a local amplifier of IL-4 mediated type-2 macrophage activation in the pulmonary immune response has been discovered in the context of a helminth infection (160). This finding could have important implications for the regulation of allergic responses.

SP-A and SP-D interact with a number of allergens and interfere with the binding of IgE antibodies thus diminishing mast cell degranulation. Interaction of inhaled allergens from the house dust mite, namely Der p 1 and Der f 1, with SP-A and SP-D have been demonstrated *in vitro* and *in vivo*. Therefore, both surfactant proteins were found to bind to dust mite extracts and purified allergens (161). Interaction of Der p 1 and Der f 1 has been shown to degrade and inactivate SP-A and SP-D, thereby interfering with the natural function of the collectins in immune defense. Proteolytic fragments of SP-A and SP-D resulting from the proteinase activity of Der p 1 were less effective in binding to Der p 1, an effect that could account for the allergenicity of these proteinase mite allergens (162).

Addition of exogenous SP-A and SP-D has been demonstrated to reduce allergic hypersensitivity. A recombinant fragment of SP-D (rfhSP-D) was recently found to be effective in supressing basophil activation and histamine release to grass pollen *Phleum pratense* Phl p *in vitro* (163). Activity of SP-A and SP-D is likewise reported to reduce allergic responses to the fungal pathogen *Aspergillus fumigatus* (Asp f). Application of exogenous SP-A, SP-D, and a recombinant rfhSP-D fragment were found to reduce Asp f-induced allergic responses *in vivo* in mice (164). The structure and composition of pulmonary surfactant is strongly related to its biophysical function in the lung. Surfactant lipid composition is strictly regulated immediately after birth to enable the process of breathing (165). The physical organization of the monolayer and interconnected bilayer phases is optimized to allow surface expansion, compression, and reducing the surface tension. Phase separation of surfactant is observed in natural lung surfactant induced by the segregation of disordered phases and ordered phases such as cholesterol-containing domains (166–168). The phase behavior is important for protein surfactant interaction, as shown for the hydrophobic SP-B (169). SP-B and SP-C are both predominantly inserted in fluid disordered-like domains of surfactant membranes (170).

Changes in lung surfactant composition can have a great impact on the biophysical surfactant function. Low amounts of bacterial LPS have been shown to disturb monolayers made from DPPC or a surfactant-like lipid mixture made from DPPC, 16:0/18:1 phosphatidylglycerol (POPG), and palmitic acid. Intercalation of LPS into the monolayer resulted in fluidization of the lipid phase, promoting early collapse and preventing the attainment of high surface pressure (171). The input of LPS carried on many allergens should therefore also be considered with respect to its effects on the biophysical surfactant function. Similar effects might be expected upon inhalation of hydrophobic allergens or lipid-binding allergens, which will naturally interact with the surfactant lipid phase due to hydrophobic interaction. An example for direct allergen surfactant interaction is reported for the aeroallergen Ole e 1 from olive tree (172). The hydrophobic N-glycosylated protein Ole e 1 showed interfacial absorption at an air-water interface, leading to an increase in surface pressure. Application of Ole e 1 to the aqueous phase under DPPC monolayer membranes led to membrane interaction of the protein resulting in an increase in surface pressure. These experiments also indicated a change in the protein orientation or conformation of the allergen upon membrane insertion, which might induce protein oligomerization or aggregation. Such structural changes are likely to affect the allergenic properties. Thus, an increase in IgE-binding and allergenicity was demonstrated for dimerized birch pollen allergen Bet v 1 (173). Another interesting aspect of the study was the observation, that Ole e 1 also interacted with 18:1(Δ9-cis) phosphatidylcholine (DOPC): sphingomyelin (SM): cholesterol model membranes, mimicking the plasma membrane of eukaryotic cells. This interaction was preferentially observed in liquid-ordered (lo) cholesterol-rich domains, indicating that membrane interaction should also be considered as a mechanism to bring hydrophobic allergens in direct contact with epithelial cells of the alveolar lining *via* interaction with lipid raft domains.

On the basis of these data, interaction of larger hydrophobic allergen/lipid aggregates such as oleosins appears to be likely considering the flexibility and connectivity of the surfactant phase and should certainly be addressed in future studies. Besides the impact on the allergic response and inflammatory regulation, surfactant interaction of lipophilic (aero)allergens leading to changes of the surfactant phase and organization is likely to have biophysical consequences that could affect lung function in general.

POTENTIAL EFFECTS OF ALLERGEN-LIPID-BINDING ON THE DEVELOPMENT OF ASTHMA AND ALLERGY

Up to now, the evidence for an association between single allergenic molecules and disease development and phenotype is epidemiological and based on the still incomplete component-resolved diagnostic tests.

For years, food storage proteins and their structural characteristics were in part responsible for the more severe class I food allergy when compared with the pollen-associated class II allergy, arguing for the fact that storage proteins are heat and digestion resistant and therefore are stable enough to induce severe symptoms.

The more we learn, however, on single allergenic molecules, the more complex the picture gets, and there is still a growing body of evidence that indeed single allergenic molecules play an important role not only in disease development but also in the shaping of phenotypes.

Via component-allergy diagnostic tests with an increasing number of single allergens from different food and inhalant allergen sources investigations on the association between clinical phenotype and sensitization profile are performed and have in some cases already resulted in the identification of potential marker allergens. Some success in this regard has been achieved for allergens from the protein families described in this manuscript. Presently, the lipocalins, Can f 1, Can f 2, and Can f 5 are available for component-resolved diagnostics as species-specific marker allergens (62). There is an association of lipocalin sensitization with asthma in children (174–176).

Whereas plant storage proteins can be used as indicators for severe food allergy, those allergens that cross-react with tree pollen in general represent sensitization that is only associated with mild to moderate symptoms. Some examples are already mentioned here in the context of nut allergy. For peanut allergy, the storage protein Ara h 2 already has been appointed as marker allergen for severe reactions. If patients have an anti-Ara h 2-IgE concentration of >42.2 kU/L, they will develop severe symptoms in 95% of cases (177). Interestingly, sensitization to peanut storage proteins only seems to occur if peanut allergy started in childhood which makes these allergens potential markers for early onset peanut allergy.

Peanut oleosins were recently shown to be marker allergens for the severity of the reaction to peanuts as well (13). Different subgroups of peanut-allergic patients were investigated: 53/74 experienced severe reactions, $n = 52$ had a genuine peanut allergy and had IgE to oleosins. Only one individual with severe reactions was a patient with a pollen-associated peanut allergy. This patient was anti-oleosin-IgE negative. All those with mild symptoms were patients with a pollen-associated peanut allergy (20). The fact that oleosins come along surrounded by phospholipids directed our research to allergen-lipid-interactions and its clinical impact.

The only oleosin-IgE-negative patient with shock to peanuts in our study was Ara h 8-IgE-positive, meaning he had a pollen-associated peanut allergy, and IgE to all other severity marker allergens were negative. This observation is supported by Glaumann et al. who described a shock in a patient who had consumed 300 g of roasted peanuts instead of a proper meal and only had Ara h 8-specific IgE antibodies (178). It is known from further examples of Bet v 1-homologs that these labile allergens, which are easily destroyed by digestion and thermal processing, may induce severe reactions when ingested in high concentrations. It is probable that in the case of Ara h 8 its association with lipids protects the allergen from immediate destruction after peanut consumption. We could show some years ago in a time course experiment, that Ara h 8 is associated with lipids which protected it for a certain period of time from enzymatic digestion (12) (see **Figure 1B**).

Mite allergy is a well-established risk factor for the development of asthma during childhood, and single mite allergens have been investigated in detail with regard to their differential influence in pathogenesis. Coming from the clinical observation, well-characterized subgroups of patients were tested for their individual sensitization profile. Investigations on the sensitization pattern to single HDM allergens, which are basically epidemiological studies, had revealed that among all those already purified and structurally characterized mite molecules, lipophilic allergens show a stronger association with asthma (6, 7). Recombinant Der p 2, Der p 5, and Der p 7 are significantly more often recognized by asthmatic patients. rDer p 5 was 3-times more often recognized by asthmatic children than by non-asthmatic children, and patients with IgE-reactivity to rDer p 5 had a probability of 85% of having HDM-asthma (7). Der p 1 or Der p 23 can predict asthma at school age (179). Cytoplasmic FABP allergens are restricted to mite bodies and are not transported *via* mite feces. Therefore, they are not important for the induction of lung/bronchial reaction due to their size but most probably responsible for HDM-induced skin reactions since they are major allergens for individuals with atopic dermatitis (180), thereby promoting organ-specificity of single allergens.

However, not only single inhalant allergens reach the broncho-alveolar cells. This seems to be the case for some food allergens as well, and not just as "pollen-associated" food allergy. Several patients with severe peanut allergy describe that they develop an allergic bronchoconstriction whenever someone in the same room opens a tin of peanuts (which was one main reason to forbid peanut snacks on airplanes where allergens were obviously distributed *via* air conditioning). Roberts et al. showed that a subgroup of food allergic children developed asthma when exposed to the aerosolized form of the food, for example while it was cooked in their presence. The authors confirmed that effect *via* bronchial provocation (181) demonstrating that foods can behave as aeroallergens. Investigations with a chewing simulator for food breakdown and flavor compound release allows deeper insight in the complex processing of food in the mouth where fluids and gas act together (182). It therefore can be hypothesized that the act of chewing peanuts drives allergens as aerosols into the bronchial system. This hypothesis is supported by the knowledge, that the delivery of the allergens takes place on the particles, containing allergens, and other chemically different substances, such as lipids. These particles become airborne and have a size small enough to reach the lungs (183). Additionally, such particles will face the milieu of the lungs, composed not only of immune cells but pulmonary surfactant as well. In this compartment, allergens/lipids come into contact with the surfactant-containing air-liquid interface that covers the epithelium of the airway mucosa underneath (153), as discussed in detail above [see section Surfactant Interaction of (Aero)allergens].

Overall, the recent evidence for the induction of pro-inflammatory responses of skin keratinocytes by peanut lipids and the facilitation of allergen penetration of the skin barrier

by lipids, together with other potential lipid-associated transport mechanisms for allergens across barriers might be an explanation for a transcutaneous sensitization to peanuts (61).

Single allergens can be used as tools for mechanistic research to clarify sensitization and allergy elicitation, thereby allowing for pathomechanistic insights with the consequence of a development of new therapeutic approaches including the development of new adjuvants and treatment vectors. Allergens with lipophilic features are missing in clinically relevant peanut extracts and most probably are underrepresented in diagnostic and therapeutic solutions of house dust mites (184), since isolation and purification methods and strategies are particularly difficult (4).

As described in more detail in the allergen-section of this manuscript, the allergen-lipid-interaction of single allergens is rather different and their effect on several cells in different compartments diverse. It is plausible that dependent on the mode of interaction of the different lipophilic allergens with lipids, these lipids may participate in the pathomechanism of asthma and allergy by (a) serving as (additional) antigen, (b) adjuvant, and/or (c) precursor for a Th2-dominated milieu.

Altogether, HDM and peanut allergy appear as good models not only for studying the effects of allergen-lipid-interactions on disease development *via* the sensitization to distinct single allergens but also for the correlation between single allergens and symptom development at specific organs (organ specificity).

AUTHOR CONTRIBUTIONS

UJ designed and wrote the manuscript. AS, KD, KS, HH, NG, and CS wrote the manuscript. CS created **Figure 1**. CS and UJ created **Table 1**.

FUNDING

Experiments on lipophilic allergens and their interaction with lipids and immune cells performed in the Research Center Borstel were funded by the Federal Ministry of Education and Research and by the German Research Foundation.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the funding of the Federal Ministry of Education and Research [German Center for Lung Research (DZL)] and of the German Research Foundation (DFG), grant JA 1007/2-1.

REFERENCES

1. Nurmatov U, van Schayck CP, Hurwitz B, Sheikh A. House dust mite avoidance measures for perennial allergic rhinitis: an updated cochrane systematic review. *Allergy* (2012) 67:158–65. doi: 10.1111/j.1398-9995.2011.02752.x
2. Leduc V, Moneret-Vautrin DA, Tzen JT, Morisset M, Guerin L, Kanny G. Identification of oleosins as major allergens in sesame seed allergic patients. *Allergy* (2006) 61:349–56. doi: 10.1111/j.1398-9995.2006.01013.x
3. Zuidmeer-Jongejan L, Fernandez-Rivas M, Winter MG, Akkerdaas JH, Summers C, Lebens A, et al. Oil body-associated hazelnut allergens including oleosins are underrepresented in diagnostic extracts but associated with severe symptoms. *Clin Transl Allergy* (2014) 4:4. doi: 10.1186/2045-7022-4-4
4. Schwager C, Kull S, Krause S, Schocker F, Petersen A, Becker WM, et al. Development of a novel strategy to isolate lipophilic allergens (oleosins) from peanuts. *PLoS ONE* (2015) 10:e0123419. doi: 10.1371/journal.pone.0123419
5. Platts-Mills TA, Rakes G, Heymann PW. The relevance of allergen exposure to the development of asthma in childhood. *J Allergy Clin Immunol.* (2000) 105(2 Pt 2):S503–8. doi: 10.1016/S0091-6749(00)90051-4
6. Weghofer M, Grote M, Resch Y, Casset A, Kneidinger M, Kopec J, et al. Identification of Der p 23, a peritrophin-like protein, as a new major dermatophagoides pteronyssinus allergen associated with the peritrophic matrix of mite fecal pellets. *J Immunol.* (2013) 190:3059–67. doi: 10.4049/jimmunol.1202288
7. Resch Y, Michel S, Kabesch M, Lupinek C, Valenta R, Vrtala S. Different IgE recognition of mite allergen components in asthmatic and nonasthmatic children. *J Allergy Clin Immunol.* (2015) 136:1083–91. doi: 10.1016/j.jaci.2015.03.024
8. Bock SA, Muñoz-Furlong A, Sampson HA. Fatalities due to anaphylactic reactions to foods. *J Allergy Clin Immunol.* (2001) 107:191–3. doi: 10.1067/mai.2001.112031
9. Worm M, Eckermann O, Doelle S, Aberer W, Beyer K, Hawranek T, et al. Triggers and treatment of anaphylaxis: an analysis of 4000 cases from Germany, Austria and Switzerland. *Deutsc Ärztebl Int.* (2014) 111:367–75. doi: 10.3238/arztebl.2014.0367
10. Grabenhenrich LB, Dölle S, Moneret-Vautrin A, Köhli A, Lange L, Spindler T, et al. Anaphylaxis in children and adolescents: The European Anaphylaxis Registry. *J Allergy Clin Immunol.* (2016) 137:1128–37. doi: 10.1016/j.jaci.2015.11.015
11. Krause S, Reese G, Randow S, Zennaro D, Quaratino D, Palazzo P, et al. Lipid transfer protein (Ara h 9) as a new peanut allergen relevant for a mediterranean allergic population. *J Allergy Clin Immunol.* (2009) 124:771–8. doi: 10.1016/j.jaci.2009.06.008
12. Petersen A, Rennert S, Kull S, Becker W-M, Notbohm H, Goldmann T, et al. Roasting and lipid binding provide allergenic and proteolytic stability to the peanut allergen Ara h 8. *Biol Chem.* (2014) 395:239–50. doi: 10.1515/hsz-2013-0206
13. Schwager C, Kull S, Behrends J, Röckendorf N, Schocker F, Frey A, et al. Peanut oleosins associated with severe peanut allergy - importance of lipophilic allergens for comprehensive allergy diagnostics. *J Allergy Clin Immunol.* (2017) 140:e1338. doi: 10.1016/j.jaci.2017.02.020
14. Hurlburt BK, Offermann LR, McBride JK, Majorek KA, Maleki SJ, Chruszcz M. Structure and function of the peanut panallergen Ara h 8. *J Biol Chem.* (2013) 288:36890–901. doi: 10.1074/jbc.M113.517797
15. Mueller GA, Gosavi RA, Krahn JM, Edwards LL, Cuneo MJ, Glesner J, et al. Der p 5 crystal structure provides insight into the group 5 dust mite allergens. *J Biol Chem.* (2010) 285:25394–401. doi: 10.1074/jbc.M110.128306
16. Rose AS, Hildebrand PW. NGL Viewer: a web application for molecular visualization. *Nucleic Acids Res.* (2015) 43:W576–9. doi: 10.1093/nar/gkv402
17. Kofler S, Asam C, Eckhard U, Wallner M, Ferreira F, Brandstetter H. Crystallographically mapped ligand binding differs in high and low IgE binding isoforms of birch pollen allergen bet v 1. *J Mol Biol.* (2012) 422:109–23. doi: 10.1016/j.jmb.2012.05.016
18. Chan SL, Ong ST, Ong SY, Chew FT, Mok YK. Nuclear magnetic resonance structure-based epitope mapping and modulation of dust mite group 13 allergen as a hypoallergen. *J Immunol.* (2006) 176:4852–60. doi: 10.4049/jimmunol.176.8.4852
19. Shin DH, Lee JY, Hwang KY, Kim KK, Suh SW. High-resolution crystal structure of the non-specific lipid-transfer protein from maize seedlings. *Structure* (1995) 3:189–99. doi: 10.1016/S0969-2126(01)00149-6
20. Jappe U, Schwager C. Relevance of lipophilic allergens in food allergy diagnosis. *Curr Allergy Asthma Rep.* (2017) 17:61. doi: 10.1007/s11882-017-0731-0

21. Fahy E, Subramaniam S, Murphy RC, Nishijima M, Raetz CR, Shimizu T, et al. Update of the LIPID MAPS comprehensive classification system for lipids. *J Lipid Res.* (2009) 50:S9–14. doi: 10.1194/jlr.R800095-JLR200
22. Bublin M, Eiwegger T, Breiteneder H. Do lipids influence the allergic sensitization process? *J Allergy Clin Immunol.* (2014) 134:521–9. doi: 10.1016/j.jaci.2014.04.015
23. Pacini E, Hesse M. Pollenkitt–its composition, forms and functions. *Flora-Morphol Distr Func Ecol Plants* (2005) 200:399–415. doi: 10.1016/j.flora.2005.02.006
24. Agea E, Russano A, Bistoni O, Mannucci R, Nicoletti I, Corazzi L, et al. Human CD1-restricted T cell recognition of lipids from pollens. *J Exp Med.* (2005) 202:295-308. doi: 10.1084/jem.20050773
25. Abós-Gracia B, del Moral MG, Lopez-Relano J, Viana-Huete V, Castro L, Villalba M, et al. *Olea europaea* pollen lipids activate invariant natural killer T cells by upregulating CD1d expression on dendritic cells. *J Allergy Clin Immunol* (2013) 131:1393–9.e5. doi: 10.1016/j.jaci.2012.11.014
26. Heydenreich B, Bellinghausen I, Konig B, Becker WM, Grabbe S, Petersen A, et al. Gram-positive bacteria on grass pollen exhibit adjuvant activity inducing inflammatory T cell responses. *Clin Exp Allergy* (2012) 42:76–84. doi: 10.1111/j.1365-2222.2011.03888.x
27. Del Moral MG, Martinez-Naves E. The role of lipids in development of allergic responses. *Immune Netw.* (2017) 17:133–43. doi: 10.4110/in.2017.17.3.133
28. King TP, Hoffman D, Lowenstein H, Marsh DG, Platts-Mills TA, Thomas W. Allergen nomenclature. *WHO/IUIS* allergen nomenclature subcommittee. *Int Arch Allergy Immunol.* (1994) 105:224–33. doi: 10.1159/000236761
29. Petersen A, Kull S, Rennert S, Becker WM, Krause S, Ernst M, et al. Peanut defensins: novel allergens isolated from lipophilic peanut extract. *J Allergy Clin Immunol.* (2015) 136:1295–301. doi: 10.1016/j.jaci.2015.04.010
30. Mogensen JE, Wimmer R, Larsen JN, Spangfort MD, Otzen DE. The major birch allergen, Bet v 1, shows affinity for a broad spectrum of physiological ligands. *J Biol Chem.* (2002) 277:23684–92. doi: 10.1074/jbc.M202065200
31. Ichikawa S, Takai T, Yashiki T, Takahashi S, Okumura K, Ogawa H, et al. Lipopolysaccharide binding of the mite allergen Der f 2. *Genes Cells* (2009) 14:1055–65. doi: 10.1111/j.1365-2443.2009.01334.x
32. Mueller GA, Edwards LL, Aloor JJ, Fessler MB, Glesner J, Pomes A, et al. The structure of the dust mite allergen Der p 7 reveals similarities to innate immune proteins. *J Allergy Clin Immunol.* (2010) 125:e904. doi: 10.1016%2Fj.jaci.2009.12.016
33. Zhao L, Chen Y, Cao Y, Kong X, Hua Y. The integral and extrinsic bioactive proteins in the aqueous extracted soybean oil bodies. *J Agric Food Chem.* (2013) 61:9727–33. doi: 10.1021/jf403327e
34. Moreno FJ, Mackie AR, Mills EN. Phospholipid interactions protect the milk allergen alpha-lactalbumin from proteolysis during *in vitro* digestion. *J Agric Food Chem.* (2005) 53:9810–6. doi: 10.1021/jf0515227
35. Markovic-Housley Z, Degano M, Lamba D, von Roepenack-Lahaye E, Clemens S, Susani M, et al. Crystal structure of a hypoallergenic isoform of the major birch pollen allergen Bet v 1 and its likely biological function as a plant steroid carrier. *J Mol Biol.* (2003) 325:123–33. doi: 10.1016/S0022-2836(02)01197-X
36. Mattila K, Renkonen R. Modelling of Bet v 1 binding to lipids. *Scand J Immunol.* (2009) 70:116–24. doi: 10.1111/j.1365-3083.2009.02277.x
37. Gülsen A, Jappe U. Lipid transfer protein sensitization in an apple-allergic patient: a case report from northern Europe. *Eur Ann Allergy Clin Immunol.* (in press). doi: 10.23822/EurAnnACI.1764-1489.63
38. Scheurer S, Schülke S. Interaction of non-specific lipid-transfer proteins with plant-derived lipids and its impact on allergic sensitization. *Front Immunol.* (2018) 9:1389. doi: 10.3389/fimmu.2018.01389
39. Petersen A, Kleine-Tebbe J, Scheurer S. Stable plant food allergens I: lipid-transfer proteins. In: Kleine-Tebbe J, Jakob T, editors *Molecular Allergy Diagnostics* (Berlin: Springer) (2017). p. 57–75.
40. Dubiela P, Aina R, Polak D, Geiselhart S, Humeniuk P, Bohle B, et al. Enhanced Pru p 3 IgE-binding activity by selective free fatty acid-interaction. *J Allergy Clin Immunol.* (2017) 140:e1710. doi: 10.1016/j.jaci.2017.06.016
41. Vassilopoulou E, Rigby N, Moreno FJ, Zuidmeer L, Akkerdaas J, Tassios I, et al. Effect of *in vitro* gastric and duodenal digestion on the allergenicity of grape lipid transfer protein. *J Allergy Clin Immunol.* (2006) 118:473–80. doi: 10.1016/j.jaci.2006.04.057
42. Vereda A, Sirvent S, Villalba M, Rodriguez R, Cuesta-Herranz J, Palomares O. Improvement of mustard (*Sinapis alba*) allergy diagnosis and management by linking clinical features and component-resolved approaches. *J Allergy Clin Immunol.* (2011) 127:1304–7. doi: 10.1016/j.jaci.2011.01.020
43. Angelina A, Sirvent S, Palladino C, Vereda A, Cuesta-Herranz J, Eiwegger T, et al. The lipid interaction capacity of Sin a 2 and Ara h 1, major mustard and peanut allergens of the cupin superfamily, endorses allergenicity. *Allergy* (2016) 71:1284–94. doi: 10.1111/all.12887
44. Mirotti L, Florsheim E, Rundqvist L, Larsson G, Spinozzi F, Leite-de-Moraes M, et al. Lipids are required for the development of Brazil nut allergy: the role of mouse and human iNKT cells. *Allergy* (2013) 68:74–83. doi: 10.1111/all.12057
45. Mueller GA, Gosavi RA, Pomes A, Wunschmann S, Moon AF, London RE, et al. Ara h 2: crystal structure and IgE binding distinguish two subpopulations of peanut allergic patients by epitope diversity. *Allergy* (2011) 66:878–85. doi: 10.1111/j.1398-9995.2010.02532.x
46. Maleki SJ, Viquez O, Jacks T, Dodo H, Champagne ET, Chung S-Y, et al. The major peanut allergen, Ara h 2, functions as a trypsin inhibitor, and roasting enhances this function. *J Allergy Clin Immunol.* (2003) 112:190–5. doi: 10.1067/mai.2003.1551
47. Maleki SJ, Chung S-Y, Champagne ET, Raufman J-P. The effects of roasting on the allergenic properties of peanut proteins. *J Allergy Clin Immunol.* (2000) 106:763–8. doi: 10.1067/mai.2000.109620
48. Chung SY, Champagne ET. Association of end-product adducts with increased IgE binding of roasted peanuts. *J Agric Food Chem.* (2001) 49:3911–6. doi: 10.1021/jf001186o
49. Johnson KL, Williams JG, Maleki SJ, Hurlburt BK, London RE, Mueller GA. Enhanced approaches for identifying amadori products: Application to peanut allergens. *J Agric Food Chem.* (2016) 64:1406–13. doi: 10.1021/acs.jafc.5b05492
50. Fu M-X, Requena JR, Jenkins AJ, Lyons TJ, Baynes JW, Thorpe SR. The advanced glycation end product, N-(carboxymethyl) lysine, is a product of both lipid peroxidation and glycoxidation reactions. *J Biol Chem.* (1996) 271:9982–6. doi: 10.1074/jbc.271.17.9982
51. Mondoulet L, Paty E, Drumare MF, Ah-Leung S, Scheinmann P, Willemot RM, et al. Influence of thermal processing on the allergenicity of peanut proteins. *J Agric Food Chem.* (2005) 53:4547–53. doi: 10.1021/jf050091p
52. Huang C-Y, Chung C-I, Lin Y-C, Hsing Y-IC, Huang AH. Oil bodies and oleosins in *Physcomitrella* possess characteristics representative of early trends in evolution. *Plant Physiol.* (2009) 150:1192–203. doi: 10.1104/pp.109.138123
53. Huang M-D, Huang AH. Bioinformatics reveal five lineages of oleosins and the mechanism of lineage evolution related to structure/function from green algae to seed plants. *Plant Physiol.* (2015) 169:453–70. doi: 10.1104/pp.15.00634
54. Weangsripanaval T, Moriyama T, Kageura T, Ogawa T, Kawada T. Dietary fat and an exogenous emulsifier increase the gastrointestinal absorption of a major soybean allergen, Gly m Bd 30K, in mice. *J Nutr.* (2005) 135:1738–44. doi: 10.1093/jn/135.7.1738
55. Chang MT, Tsai TR, Lee CY, Wei YS, Chen YJ, Chen CR, et al. Elevating bioavailability of curcumin via encapsulation with a novel formulation of artificial oil bodies. *J Agri Food Chem.* (2013) 61:9666–71. doi: 10.1021/jf4019195
56. Li W, Yang J, Cai J, Wang H, Tian H, Huang J, et al. Oil body-bound oleosin-rhFGF-10: a novel drug delivery system that improves skin penetration to accelerate wound healing and hair growth in mice. *Int J Molecul Sci.* (2017) 18:2177. doi: 10.3390/ijms18102177
57. Chen Y, Zhao L, Kong X, Zhang C, Hua Y. The properties and the related protein behaviors of oil bodies in soymilk preparation. *Eur Food Res Technol.* (2014) 239:463–71. doi: 10.1007/s00217-014-2239-3
58. Cao Y, Zhao L, Ying Y, Kong X, Hua Y, Chen Y. The characterization of soybean oil body integral oleosin isoforms and the effects of alkaline pH on them. *Food Chem.* (2015) 177:288–94. doi: 10.1016/j.foodchem.2015.01.052
59. Olszewski A, Pons L, Moutete F, Aimone-Gastin I, Kanny G, Moneret-Vautrin DA, et al. Isolation and characterization of proteic allergens in refined peanut oil. *Clin Exp Allergy* (1998) 28:850–9. doi: 10.1046/j.1365-2222.1998.00325.x

60. Ring J, Mohrenschlager M. Allergy to peanut oil-clinically relevant? *J Eur Acad Dermatol Venereol.* (2007) 21:452–5. doi: 10.1111/j.1468-3083.2006.02133.x
61. Palladino C, Narzt MS, Bublin M, Schreiner M, Humeniuk P, Gschwandtner M, et al. Peanut lipids display potential adjuvanticity by triggering a pro-inflammatory response in human keratinocytes. *Allergy* (2018) 73:1746–9. doi: 10.1111/all.13475
62. Matricardi P, Kleine-Tebbe J, Hoffmann H, Valenta R, Hilger C, Hofmaier S, et al. EAACI molecular allergology user's guide. *Pediatr Allergy Immunol.* (2016) 27, 1–250. doi: 10.1111/pai.12563
63. Hilger C, Kleine-Tebbe J, van Hage M. Molecular diagnostics in allergy to mammals. In: Kleine-Tebbe J, Jakob T, editors *Molecular Allergy Diagnostics* (Berlin: Springer) (2017). p. 363–79. doi: 10.1007/978-3-319-42499-6_19
64. Virtanen T, Kinnunen T. Adaptive immunity as a determinant of allergenicity. *J Allergy Clin Immunol.* (2009) 124:171–2. doi: 10.1016/j.jaci.2009.04.011
65. Herre J, Gronlund H, Brooks H, Hopkins L, Waggoner L, Murton B, et al. Allergens as immunomodulatory proteins: the cat dander protein Fel d 1 enhances TLR activation by lipid ligands. *J Immunol.* (2013) 191:1529–35. doi: 10.4049/jimmunol.1300284
66. Osterlund C, Gronlund H, Polovic N, Sundstrom S, Gafvelin G, Bucht A. The non-proteolytic house dust mite allergen Der p 2 induce NF-kappaB and MAPK dependent activation of bronchial epithelial cells. *Clin Exp Allergy* (2009) 39:1199–208. doi: 10.1111/j.1365-2222.2009.03284.x
67. Trompette A, Divanovic S, Visintin A, Blanchard C, Hegde RS, Madan R, et al. Allergenicity resulting from functional mimicry of a Toll-like receptor complex protein. *Nature* (2009) 457:585–8. doi: 10.1038/nature07548
68. Pulsawat P, Theeraapisakkun M, Nony E, Le Mignon M, Jain K, Buaklin A, et al. Characterization of the house dust mite allergen Der p 21 produced in Pichia pastoris. *Protein Exp Purif.* (2014) 101:8–13. doi: 10.1016/j.pep.2014.05.001
69. Shimazu R, Akashi S, Ogata H, Nagai Y, Fukudome K, Miyake K, et al. MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4. *J Exp Med.* (1999) 189:1777–82. doi: 10.1084/jem.189.11.1777
70. Satitsuksanoa P, Kennedy M, Gilis D, Le Mignon M, Suratannon N, Soh WT, et al. The minor house dust mite allergen Der p 13 is a fatty acid-binding protein and an activator of a TLR2-mediated innate immune response. *Allergy* (2016) 71:1425–34. doi: 10.1111/all.12899
71. Zheng Y. Fatty acid-binding proteins at a glance. *Protein Pept Lett.* (2014) 21:572–7. doi: 10.2174/0929866521061404251246 28
72. Ganfornina MD, Gutierrez G, Bastiani M, Sanchez D. A phylogenetic analysis of the lipocalin protein family. *Mol Biol Evol.* (2000) 17:114–26. doi: 10.1093/oxfordjournals.molbev.a026224
73. Schaap FG, van der Vusse GJ, Glatz JF. Evolution of the family of intracellular lipid binding proteins in vertebrates. *Mol Cell Biochem.* (2002) 239:69–77. doi: 10.1023/A:1020519011939
74. Thomas WR. Hierarchy and molecular properties of house dust mite allergens. *Allergol Int.* (2015) 64:304–11. doi: 10.1016/j.alit.2015.05.004
75. Storch J, Thumser AE. Tissue-specific functions in the fatty acid-binding protein family. *J Biol Chem.* (2010) 285:32679–83. doi: 10.1074/jbc.R110.135210
76. Kawooya JK, Keim PS, Ryan RO, Shapiro JP, Samaraweera P, Law JH. Insect apolipophorin III. Purification and properties. *J Biol Chem.* (1984) 259:10733–7.
77. Epton MJ, Dilworth RJ, Smith W, Thomas WR. Sensitisation to the lipid-binding apolipophorin allergen Der p 14 and the peptide Mag-1. *Int Arch Allergy Immunol.* (2001) 124:57–60. doi: 10.1159/000053668
78. Fujikawa A, Uchida K, Yanagidani A, Kawamoto S, Aki T, Shigeta S, et al. Altered antigenicity of M-177, a 177-kDa allergen from the house dust mite *Dermatophagoides farinae*, in stored extract. *Clin Exp Allergy* (1998) 28:1549–58. doi: 10.1046/j.1365-2222.1998.00433.x
79. Yoshimoto T, Bendelac A, Watson C, Hu-Li J, Paul WE. Role of NK1.1+ T cells in a TH2 response and in immunoglobulin E production. *Science* (1995) 270:1845–7. doi: 10.1126/science.270.5243.1845
80. Meyer E, DeKruyff R, Umetsu D. iNKT cells in allergic disease. In: Moody, editor *T Cell Activation by CD1 and Lipid Antigens* (Berlin: Springer) (2007). p. 269–91. doi: 10.1007/978-3-540-69511-0_11
81. Stock P, Akbari O. Recent advances in the role of NKT cells in allergic diseases and asthma. *Curr Allergy Asthma Rep.* (2008) 8:165–70. doi: 10.1007/s11882-008-0027-5
82. Albacker LA, Chaudhary V, Chang YJ, Kim HY, Chuang YT, Pichavant M, et al. Invariant natural killer T cells recognize a fungal glycosphingolipid that can induce airway hyperreactivity. *Nat Med.* (2013) 19:1297–304. doi: 10.1038/nm.3321
83. Tordesillas L, Cubells-Baeza N, Gomez-Casado C, Berin C, Esteban V, Barcik W, et al. Mechanisms underlying induction of allergic sensitization by Pru p 3. *Clin Exp Allergy* (2017) 47:1398–408. doi: 10.1111/cea.12962
84. Jyonouchi S, Abraham V, Orange JS, Spergel JM, Gober L, Dudek E, et al. Invariant natural killer T cells from children with versus without food allergy exhibit differential responsiveness to milk-derived sphingomyelin. *J Allergy Clin Immunol.* (2011) 128:e113. doi: 10.1016/j.jaci.2011.02.026
85. Cianferoni A, Saltzman R, Saretta F, Barni S, Dudek E, Kelleher M, et al. Invariant natural killer cells change after an oral allergy desensitization protocol for cow's milk. *Clin Exp Allergy* (2017) 47:1390–7. doi: 10.1111/cea.12975
86. Rajavelu P, Rayapudi M, Moffitt M, Mishra A, Mishra A. Significance of para-esophageal lymph nodes in food or aeroallergen-induced iNKT cell-mediated experimental eosinophilic esophagitis. *Am J Physiol Gastrointest Liver Physiol.* (2012) 302:G645–54. doi: 10.1152/ajpgi.00223.2011
87. Moody DB. TLR gateways to CD1 function. *Nat Immunol.* (2006) 7:811–7. doi: 10.1038/ni1368
88. Skold M, Xiong X, Illarionov PA, Besra GS, Behar SM. Interplay of cytokines and microbial signals in regulation of CD1d expression and NKT cell activation. *J Immunol.* (2005) 175:3584–93. doi: 10.4049/jimmunol.175.6.3584
89. Salio M, Speak AO, Shepherd D, Polzella P, Illarionov PA, Veerapen N, et al. Modulation of human natural killer T cell ligands on TLR-mediated antigen-presenting cell activation. *Proc Natl Acad Sci USA.* (2007) 104:20490–5. doi: 10.1073/pnas.0710145104
90. Askenase PW, Itakura A, Leite-de-Moraes MC, Lisbonne M, Roongapinun S, Goldstein, DR, et al. TLR-dependent IL-4 production by invariant Valpha14+Jalpha18+ NKT cells to initiate contact sensitivity *in vivo*. *J Immunol.* (2005) 175:6390–401. doi: 10.4049/jimmunol.175.10.6390
91. Yoshimoto T. The hunt for the source of primary interleukin-4: how we discovered that natural killer t cells and basophils determine T helper type 2 cell differentiation *in vivo*. *Front Immunol.* (2018) 9:716. doi: 10.3389/fimmu.2018.00716
92. Vincent MS, Leslie DS, Gumperz JE, Xiong X, Grant EP, Brenner MB. CD1-dependent dendritic cell instruction. *Nat Immunol.* (2002) 3:1163–8. doi: 10.1038/ni851
93. Aliprantis AO, Yang RB, Mark MR, Suggett S, Devaux B, Radolf JD, et al. Cell activation and apoptosis by bacterial lipoproteins through toll-like receptor-2. *Science* (1999) 285:736–9. doi: 10.1126/science.285.5428.736
94. Takeuchi O, Hoshino K, Kawai T, Sanjo H, Takada H, Ogawa T, et al. Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. *Immunity* (1999) 11:443–51. doi: 10.1016/S1074-7613(00)80119-3
95. Poltorak A, He X, Smirnova I, Liu MY, Van Huffel C, Du X, et al. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science* (1998) 282:2085–8. doi: 10.1126/science.282.5396.2085
96. Williams LK, Ownby DR, Maliarik MJ, Johnson CC. The role of endotoxin and its receptors in allergic disease. *Ann Allergy Asthma Immunol.* (2005) 94:323–32. doi: 10.1016/S1081-1206(10)60983-0
97. Bezemer GF, Sagar S, van Bergenhenegouwen J, Georgiou NA, Garssen J, Kraneveld AD, et al. Dual role of Toll-like receptors in asthma and chronic obstructive pulmonary disease. *Pharmacol Rev.* (2012) 64:337–58. doi: 10.1124/pr.111.004622
98. Dong Z, Xiong L, Zhang W, Gibson PG, Wang T, Lu Y, et al. Holding the inflammatory system in check: TLRs and their targeted therapy in asthma. *Med Inflamm.* (2016) 2016:2180417. doi: 10.1155/2016/2180417
99. Delayre-Orthez C, de Blay F, Frossard N, Pons F. Dose-dependent effects of endotoxins on allergen sensitization and challenge in the mouse. *Clin Exp Allergy* (2004) 34:1789–95. doi: 10.1111/j.1365-2222.2004.02082.x

100. Fuchs B, Braun A. Modulation of asthma and allergy by addressing toll-like receptor 2. *J Occup Med Toxicol. (2008) 3(Suppl. 1):S5.* doi: 10.1186/1745-6673-3-S1-S5
101. Debarry J, Hanuszkiewicz A, Stein K, Holst O, Heine H. The allergy-protective properties of Acinetobacter lwoffii F78 are imparted by its lipopolysaccharide. *Allergy* (2010) 65:690–7. doi: 10.1111/j.1398-9995.2009.02253.x
102. Brightbill HD, Libraty DH, Krutzik SR, Yang RB, Belisle JT, Bleharski JR, et al. Host defense mechanisms triggered by microbial lipoproteins through toll-like receptors. *Science* (1999) 285:732–6. doi: 10.1126/science.285.5428.732
103. Takeuchi O, Kawai T, Muhlradt PF, Morr M, Radolf JD, Zychlinsky A, et al. Discrimination of bacterial lipoproteins by Toll-like receptor 6. *Int Immunol.* (2001) 13:933–40. doi: 10.1093/intimm/13.7.933
104. Takeuchi O, Sato S, Horiuchi T, Hoshino K, Takeda K, Dong Z, et al. Cutting edge: role of Toll-like receptor 1 in mediating immune response to microbial lipoproteins. *J Immunol.* (2002) 169:10–4. doi: 10.4049/jimmunol.169.1.10
105. Kang JY, Nan X, Jin MS, Youn SJ, Ryu YH, Mah S, et al. Recognition of lipopeptide patterns by Toll-like receptor 2-Toll-like receptor 6 heterodimer. *Immunity* (2009) 31:873–84. doi: 10.1016/j.immuni.2009.09.018
106. Jin MS, Kim SE, Heo JY, Lee ME, Kim HM, Paik SG, et al. Crystal structure of the TLR1-TLR2 heterodimer induced by binding of a tri-acylated lipopeptide. *Cell* (2007) 130:1071–82. doi: 10.1016/j.cell.2007.09.008
107. Farhat K, Riekenberg S, Heine H, Debarry J, Lang R, Mages J, et al. Heterodimerization of TLR2 with TLR1 or TLR6 expands the ligand spectrum but does not lead to differential signaling. *J Leukoc Biol.* (2008) 83:692–701. doi: 10.1189/jlb.0807586
108. Galanos C, Luderitz O, Rietschel ET, Westphal O, Brade H, Brade L, et al. Synthetic and natural Escherichia coli free lipid A express identical endotoxic activities. *Eur J Biochem.* (1985) 148:1–5. doi: 10.1111/j.1432-1033.1985.tb08798.x
109. Park BS, Song DH, Kim HM, Choi BS, Lee H, Lee JO. The structural basis of lipopolysaccharide recognition by the TLR4-MD-2 complex. *Nature* (2009) 458:1191–5. doi: 10.1038/nature07830
110. Ohto U, Fukase K, Miyake K, Shimizu T. Structural basis of species-specific endotoxin sensing by innate immune receptor TLR4/MD-2. *Proc Natl Acad Sci USA.* (2012) 109:7421–6. doi: 10.1073/pnas.1201193109
111. Lien E, Means TK, Heine H, Yoshimura A, Kusumoto S, Fukase K, et al. Toll-like receptor 4 imparts ligand-specific recognition of bacterial lipopolysaccharide. *J Clin Invest.* (2000) 105:497–504. doi: 10.1172/JCI8541
112. Kawai T, Adachi O, Ogawa T, Takeda K, Akira S. Unresponsiveness of MyD88-deficient mice to endotoxin. *Immunity* (1999) 11:115–22. doi: 10.1016/S1074-7613(00)80086-2
113. Hoebe K, Du X, Georgel P, Janssen E, Tabeta K, Kim SO, et al. Identification of Lps2 as a key transducer of MyD88-independent TIR signalling. *Nature* (2003) 424:743–8. doi: 10.1038/nature01889
114. Yamamoto M, Sato S, Hemmi H, Hoshino K, Kaisho T, Sanjo H, et al. Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. *Science* (2003) 301:640–3. doi: 10.1126/science.1087262
115. Dubin W, Martin TR, Swoveland P, Leturcq DJ, Moriarty AM, Tobias PS, et al. Asthma and endotoxin: lipopolysaccharide-binding protein and soluble CD14 in bronchoalveolar compartment. *Am J Physiol.* (1996) 270:L736–44. doi: 10.1152/ajplung.1996.270.5.L736
116. Strohmeier GR, Walsh JH, Klings ES, Farber HW, Cruikshank WW, Center DM, et al. Lipopolysaccharide binding protein potentiates airway reactivity in a murine model of allergic asthma. *J Immunol.* (2001) 166:2063–70. doi: 10.4049/jimmunol.166.3.2063
117. Bufe A, Holst O. LPS-binding protein as a target molecule in allergy and asthma. *J Allergy Clin Immunol.* (2004) 114:583–5. doi: 10.1016/j.jaci.2004.06.034
118. Schumann RR, Kirschning CJ, Unbehaun A, Aberle HP, Knope HP, Lamping N, et al. The lipopolysaccharide-binding protein is a secretory class 1 acute-phase protein whose gene is transcriptionally activated by APRF/STAT/3 and other cytokine-inducible nuclear proteins. *Mol Cell Biol.* (1996) 16:3490–503. doi: 10.1128/MCB.16.7.3490
119. Vreugdenhil AC, Dentener MA, Snoek AM, Greve JW, Buurman WA. Lipopolysaccharide binding protein and serum amyloid A secretion by human intestinal epithelial cells during the acute phase response. *J Immunol.* (1999) 163:2792–8.
120. Dentener MA, Vreugdenhil AC, Hoet PH, Vernooy JH, Nieman FH, Heumann D, et al. Production of the acute-phase protein lipopolysaccharide-binding protein by respiratory type II epithelial cells: implications for local defense to bacterial endotoxins. *Am J Respir Cell Mol Biol.* (2000) 23:146–53. doi: 10.1165/ajrcmb.23.2.3855
121. Bingle CD, Bingle L. Characterisation of the human plunc gene, a gene product with an upper airways and nasopharyngeal restricted expression pattern. *Biochim Biophys Acta* (2000) 1493:363–7. doi: 10.1016/S0167-4781(00)00196-2
122. Bingle CD, Craven CJ. Meet the relatives: a family of BPI- and LBP-related proteins. *Trends Immunol.* (2004) 25:53–5. doi: 10.1016/j.it.2003.11.007
123. Elsbach P, Weiss J. Role of the bactericidal/permeability-increasing protein in host defence. *Curr Opin Immunol.* (1998) 10:45–9. doi: 10.1016/S0952-7915(98)80030-7
124. Schumann RR, Leong SR, Flaggs GW, Gray PW, Wright SD, Mathison JC, et al. Structure and function of lipopolysaccharide binding protein. *Science* (1990) 249:1429–31. doi: 10.1126/science.2402637
125. Alva V, Lupas AN. The TULIP superfamily of eukaryotic lipid-binding proteins as a mediator of lipid sensing and transport. *Biochim Biophys Acta* (2016) 1861:913–23. doi: 10.1016/j.bbalip.2016.01.016
126. Wong LH, Levine TP. Tubular lipid binding proteins (TULIPs) growing everywhere. *Biochim Biophys Acta* (2017) 1864:1439–49. doi: 10.1016/j.bbamcr.2017.05.019
127. Qiu X, Mistry A, Ammirati MJ, Chrunyk BA, Clark RW, Cong Y, et al. Crystal structure of cholesteryl ester transfer protein reveals a long tunnel and four bound lipid molecules. *Nat Struct Mol Biol.* (2007) 14:106–13. doi: 10.1038/nsmb1197
128. Schroder NW, Heine H, Alexander C, Manukyan M, Eckert J, Hamann L, et al. Lipopolysaccharide binding protein binds to triacylated and diacylated lipopeptides and mediates innate immune responses. *J Immunol.* (2004) 173:2683–91. doi: 10.4049/jimmunol.173.4.2683
129. Manukyan M, Triantafilou K, Triantafilou M, Mackie A, Nilsen N, Espevik T, et al. Binding of lipopeptide to CD14 induces physical proximity of CD14, TLR2 and TLR1. *Eur J Immunol.* (2005) 35:911–21. doi: 10.1002/eji.200425336
130. Ryu JK, Kim SJ, Rah SH, Kang JI, Jung HE, Lee D, et al. Reconstruction of LPS transfer cascade reveals structural determinants within LBP, CD14, and TLR4-MD2 for efficient LPS recognition and transfer. *Immunity* (2017) 46:38–50. doi: 10.1016/j.immuni.2016.11.007
131. Fan MH, Klein RD, Steinstraesser L, Merry AC, Nemzek JA, Remick DG, et al. An essential role for lipopolysaccharide-binding protein in pulmonary innate immune responses. *Shock* (2002) 18:248–54. doi: 10.1097/00024382-200209000-00008
132. Branger J, Florquin S, Knapp S, Leemans JC, Pater JM, Speelman P, et al. LPS-binding protein-deficient mice have an impaired defense against Gram-negative but not Gram-positive pneumonia. *Int Immunol.* (2004) 16:1605–11. doi: 10.1093/intimm/dxh161
133. Beamer LJ, Carroll SF, Eisenberg D. Crystal structure of human BPI and two bound phospholipids at 2.4 angstrom resolution. *Science* (1997) 276:1861–4. doi: 10.1126/science.276.5320.1861
134. Eckert JK, Kim YJ, Kim JI, Gurtler K, Oh DY, Sur S, et al. The crystal structure of lipopolysaccharide binding protein reveals the location of a frequent mutation that impairs innate immunity. *Immunity* (2013) 39:647–60. doi: 10.1016/j.immuni.2013.09.005
135. Schromm AB, Brandenburg K, Rietschel ET, Flad HD, Carroll SF, Seydel U. Lipopolysaccharide-binding protein mediates CD14-independent intercalation of lipopolysaccharide into phospholipid membranes. *FEBS Lett.* (1996) 399:267–71. doi: 10.1016/S0014-5793(96)01338-5
136. Yu B, Hailman E, Wright SD. Lipopolysaccharide binding protein and soluble CD14 catalyze exchange of phospholipids. *J Clin Invest.* (1997) 99:315–24. doi: 10.1172/JCI119160
137. Mueller M, Brandenburg K, Dedrick R, Schromm AB, Seydel U. Phospholipids inhibit lipopolysaccharide (LPS)-induced cell activation: a role for LPS-binding protein. *J Immunol.* (2005) 174:1091–6. doi: 10.4049/jimmunol.174.2.1091
138. Kuronuma K, Mitsuzawa H, Takeda K, Nishitani C, Chan ED, Kuroki Y, et al. Anionic pulmonary surfactant phospholipids inhibit inflammatory responses from alveolar macrophages and U937 cells by binding the

lipopolysaccharide-interacting proteins CD14 and MD-2. *J Biol Chem.* (2009) 284:25488–500. doi: 10.1074/jbc.M109.040832
139. Numata M, Kandasamy P, Voelker DR. Anionic pulmonary surfactant lipid regulation of innate immunity. *Exp Rev Respir Med.* (2012) 6:243–6. doi: 10.1586/ers.12.21
140. Spengler D, Winoto-Morbach S, Kupsch S, Vock C, Blochle K, Frank S, et al. Novel therapeutic roles for surfactant-inositols and -phosphatidylglycerols in a neonatal piglet ARDS model: a translational study. *Am J Physiol Lung Cell Mol Physiol.* (2018) 314:L32–53. doi: 10.1152/ajplung.00128.2017
141. Bochkov VN, Kadl A, Huber J, Gruber F, Binder BR, Leitinger N. Protective role of phospholipid oxidation products in endotoxin-induced tissue damage. *Nature* (2002) 419:77–81. doi: 10.1038/nature01023
142. Chu LH, Indramohan M, Ratsimandresey RA, Gangopadhyay A, Morris EP, Monack DM, et al. The oxidized phospholipid oxPAPC protects from septic shock by targeting the non-canonical inflammasome in macrophages. *Nat Commun.* (2018) 9:996. doi: 10.1038/s41467-018-03409-3
143. Yu B, Benning C. Anionic lipids are required for chloroplast structure and function in arabidopsis. *Plant J.* (2003) 36:762–70. doi: 10.1046/j.1365-313X.2003.01918.x
144. Kopp F, Kupsch S, Schromm AB. Lipopolysaccharide-binding protein is bound and internalized by host cells and colocalizes with LPS in the cytoplasm: Implications for a role of LBP in intracellular LPS-signaling. *Biochim Biophys Acta* (2016) 1863:660–72. doi: 10.1016/j.bbamcr.2016.01.015
145. Gakhar L, Bartlett JA, Penterman J, Mizrachi D, Singh PK, Mallampalli RK, et al. PLUNC is a novel airway surfactant protein with anti-biofilm activity. *PLoS ONE* (2010) 5:e9098. doi: 10.1371/journal.pone.0009098
146. Seshadri S, Lin DC, Rosati M, Carter RG, Norton JE, Suh L, et al. Reduced expression of antimicrobial PLUNC proteins in nasal polyp tissues of patients with chronic rhinosinusitis. *Allergy* (2012) 67:920–8. doi: 10.1111/j.1398-9995.2012.02848.x
147. Lukinskiene L, Liu Y, Reynolds SD, Steele C, Stripp BR, Leikauf GD, et al. Antimicrobial activity of PLUNC protects against *Pseudomonas aeruginosa* infection. *J Immunol.* (2011) 187:382–90. doi: 10.4049/jimmunol.1001769
148. Liu Y, Bartlett JA, Di ME, Bomberger JM, Chan YR, Gakhar L, et al. SPLUNC1/BPIFA1 contributes to pulmonary host defense against *Klebsiella pneumoniae* respiratory infection. *Am J Pathol.* (2013) 182:1519–31. doi: 10.1016/j.ajpath.2013.01.050
149. Ghafouri B, Kihlstrom E, Tagesson C, Lindahl M. PLUNC in human nasal lavage fluid: multiple isoforms that bind to lipopolysaccharide. *Biochim Biophys Acta* (2004) 1699:57–63. doi: 10.1016/S1570-9639(04)00003-2
150. Ning F, Wang C, Berry KZ, Kandasamy P, Liu H, Murphy RC, et al. Structural characterization of the pulmonary innate immune protein SPLUNC1 and identification of lipid ligands. *FASEB J.* (2014) 28:5349–60. doi: 10.1096/fj.14-259291
151. McDonald RE, Fleming RI, Beeley JG, Bovell DL, Lu JR, Zhao X, et al. Latherin: a surfactant protein of horse sweat and saliva. *PLoS ONE* (2009) 4:e5726. doi: 10.1371/journal.pone.0005726
152. Vance SJ, McDonald RE, Cooper A, Smith BO, Kennedy MW. The structure of latherin, a surfactant allergen protein from horse sweat and saliva. *J R Soc Interf.* (2013) 10:20130453. doi: 10.1098/rsif.2013.0453
153. Winkler C, Hohlfeld JM. Surfactant and allergic airway inflammation. *Swiss Med Wkly* (2013) 143:w13818. doi: 10.4414/smw.2013.13818
154. Perez-Gil J. Structure of pulmonary surfactant membranes and films: the role of proteins and lipid-protein interactions. *Biochim Biophys Acta* (2008) 1778:1676–95. doi: 10.1016/j.bbamem.2008.05.003
155. Hamvas A, Nogee LM, deMello DE, Cole FS. Pathophysiology and treatment of surfactant protein-B deficiency. *Biol Neonate* (1995) 67(Suppl. 1):18–31. doi: 10.1159/000244204
156. Haczku A, Atochina EN, Tomer Y, Chen H, Scanlon ST, Russo S, et al. *Aspergillus fumigatus*-induced allergic airway inflammation alters surfactant homeostasis and lung function in BALB/c mice. *Am J Respir Cell Mol Biol.* (2001) 25:45–50. doi: 10.1165/ajrcmb.25.1.4391
157. Wright JR. Immunoregulatory functions of surfactant proteins. *Nat Rev Immunol.* (2005) 5:58–68. doi: 10.1038/nri1528
158. Moulakakis C, Adam S, Seitzer U, Schromm AB, Leitges M, Stamme C. Surfactant protein A activation of atypical protein kinase C zeta in IkappaB-alpha-dependent anti-inflammatory immune regulation. *J Immunol.* (2007) 179:4480–91. doi: 10.4049/jimmunol.179.7.4480
159. Sorensen GL, Husby S, Holmskov U. Surfactant protein A and surfactant protein D variation in pulmonary disease. *Immunobiology* (2007) 212:381–416. doi: 10.1016/j.imbio.2007.01.003
160. Minutti CM, Jackson-Jones LH, Garcia-Fojeda B, Knipper JA, Sutherland TE, Logan N, et al. Local amplifiers of IL-4Ralpha-mediated macrophage activation promote repair in lung and liver. *Science* (2017) 356:1076–80. doi: 10.1126/science.aaj2067
161. Wang JY, Kishore U, Lim BL, Strong P, Reid KB. Interaction of human lung surfactant proteins A and D with mite (*Dermatophagoides pteronyssinus*) allergens. *Clin Exp Immunol.* (1996) 106:367–73. doi: 10.1046/j.1365-2249.1996.d01-838.x
162. Deb R, Shakib F, Reid K, Clark H. Major house dust mite allergens *Dermatophagoides pteronyssinus* 1 and *Dermatophagoides farinae* 1 degrade and inactivate lung surfactant proteins A and D. *J Biol Chem.* (2007) 282:36808–19. doi: 10.1074/jbc.M702336200
163. Qaseem AS, Singh I, Pathan AA, Layhadi JA, Parkin R, Alexandra F, et al. A recombinant fragment of human surfactant protein d suppresses basophil activation and T-Helper Type 2 and B-cell responses in grass pollen-induced allergic inflammation. *Am J Respir Crit Care Med.* (2017) 196:1526–34. doi: 10.1164/rccm.201701-0225OC
164. Madan T, Kishore U, Singh M, Strong P, Clark H, Hussain EM, et al. Surfactant proteins A and D protect mice against pulmonary hypersensitivity induced by *Aspergillus fumigatus* antigens and allergens. *J Clin Invest.* (2001) 107:467–75. doi: 10.1172/JCI10124
165. Dautel SE, Kyle JE, Clair G, Sontag RL, Weitz KK, Shukla AK, et al. Lipidomics reveals dramatic lipid compositional changes in the maturing postnatal lung. *Sci Rep.* (2017) 7:40555. doi: 10.1038/srep40555
166. Nag K, Pao JS, Harbottle RR, Possmayer F, Petersen NO, Bagatolli LA. Segregation of saturated chain lipids in pulmonary surfactant films and bilayers. *Biophys J.* (2002) 82:2041–51. doi: 10.1016/S0006-3495(02)75552-5
167. Bernardino de la Serna J, Oradd G, Bagatolli LA, Simonsen AC, Marsh D, Lindblom, G, et al. Segregated phases in pulmonary surfactant membranes do not show coexistence of lipid populations with differentiated dynamic properties. *Biophys J.* (2009) 97:1381–9. doi: 10.1016/j.bpj.2009.06.040
168. Casals C, Canadas O. Role of lipid ordered/disordered phase coexistence in pulmonary surfactant function. *Biochim Biophys Acta* (2012) 1818:2550–62. doi: 10.1016/j.bbamem.2012.05.024
169. Seifert M, Breitenstein D, Klenz U, Meyer MC, Galla HJ. Solubility versus electrostatics: what determines lipid/protein interaction in lung surfactant. *Biophys J.* (2007) 93:1192–203. doi: 10.1529/biophysj.107.106765
170. Bernardino de la Serna J, Perez-Gil J, Simonsen AC, Bagatolli LA. Cholesterol rules: direct observation of the coexistence of two fluid phases in native pulmonary surfactant membranes at physiological temperatures. *J Biol Chem.* (2004) 279:40715–22. doi: 10.1074/jbc.M404648200
171. Canadas O, Keough KM, Casals C. Bacterial lipopolysaccharide promotes destabilization of lung surfactant-like films. *Biophys J.* (2011) 100:108–16. doi: 10.1016/j.bpj.2010.11.028
172. Lopez-Rodriguez JC, Barderas R, Echaide M, Perez-Gil J, Villalba M, Batanero E, et al. Surface activity as a crucial factor of the biological actions of Ole e 1, the main aeroallergen of olive tree (*Olea europaea*) pollen. *Langmuir* (2016) 32:11055–62. doi: 10.1021/acs.langmuir.6b02831
173. Scholl I, Kalkura N, Shedziankova Y, Bergmann A, Verdino P, Knittelfelder R, et al. Dimerization of the major birch pollen allergen Bet v 1 is important for its *in vivo* IgE-cross-linking potential in mice. *J Immunol.* (2005) 175:6645–50. doi: 10.4049/jimmunol.175.10.6645
174. Hilger C, Kuehn A, Hentges F. Animal lipocalin allergens. *Curr Allergy Asthma Rep.* (2012) 12:438–47. doi: 10.1007/s11882-012-0283-2
175. Hilger C, Swiontek K, Arumugam K, Lehners C, Hentges F. Identification of a new major dog allergen highly cross-reactive with Fel d 4 in a population of cat- and dog-sensitized patients. *J Allergy Clin Immunol.* (2012) 129:1149–51. doi: 10.1016/j.jaci.2011.10.017
176. Nordlund B, Konradsen JR, Kull I, Borres MP, Onell A, Hedlin G, et al. IgE antibodies to animal-derived lipocalin, kallikrein and secretoglobin are

markers of bronchial inflammation in severe childhood asthma. *Allergy* (2012) 67:661–9. doi: 10.1111/j.1398-9995.2012.02797.x

177. Beyer K, Grabenhenrich L, Haertl M, Beder A, Kalb B, Ziegert M, et al. Predictive values of component-specific IgE for the outcome of peanut and hazelnut food challenges in children. *Allergy* (2015) 70:90–8. doi: 10.1111/all.12530
178. Glaumann S, Nopp A, Johansson SGO, Borres MP, Lilja G, Nilsson C. Anaphylaxis to peanuts in a 16-year-old girl with birch pollen allergy and with monosensitization to Ara h 8. *J Allergy Clin Immunol.* (2013) 1:698–9. doi: 10.1016/j.jaip.2013.08.010
179. Posa D, Perna S, Resch Y, Lupinek C, Panetta V, Hofmaier S, et al. Evolution and predictive value of IgE responses toward a comprehensive panel of house dust mite allergens during the first 2 decades of life. *J Allergy Clin Immunol.* (2017) 139:e548. doi: 10.1016/j.jaci.2016.08.014
180. Banerjee S, Resch Y, Chen KW, Swoboda I, Focke-Tejkl M, Blatt K, et al. Der p 11 is a major allergen for house dust mite-allergic patients suffering from atopic dermatitis. *J Invest Dermatol.* (2015) 135:102–9. doi: 10.1038/jid.2014.271
181. Roberts G, Golder N, Lack G. Bronchial challenges with aerosolized food in asthmatic, food-allergic children. *Allergy* (2002) 57:713–7. doi: 10.1034/j.1398-9995.2002.03366.x
182. Salles C, Tarrega A, Mielle P, Maratray J, Gorria P, Liaboeuf J, et al. Development of a chewing simulator for food breakdown and the analysis of *in vitro* flavor compound release in a mouth environment. *J Food Eng.* (2007) 82:189–98. doi: 10.1016/j.jfoodeng.2007.02.008
183. Woodfolk JA, Commins SP, Schuyler AJ, Erwin EA, Platts-Mills TA. Allergens, sources, particles, and molecules: why do we make IgE responses? *Allergol Int.* (2015) 64:295–303. doi: 10.1016/j.alit.2015.06.001
184. Casset A, Mari A, Purohit A, Resch Y, Weghofer M, Ferrara R, et al. Varying allergen composition and content affects the *in vivo* allergenic activity of commercial *Dermatophagoides pteronyssinus* extracts. *Int Arch Allergy Immunol.* (2012) 159:253–62. doi: 10.1159/000337654

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

ORIGINAL RESEARCH
published: 03 May 2019
doi: 10.3389/fimmu.2019.00974

Lipid Mediators From Timothy Grass Pollen Contribute to the Effector Phase of Allergy and Prime Dendritic Cells for Glycolipid Presentation

*Nestor González Roldán[1], Regina Engel[1], Sylvia Düpow[1], Katharina Jakob[1], Frauke Koops[2], Zane Orinska[2], Claire Vigor[3], Camille Oger[3], Jean-Marie Galano[3], Thierry Durand[3], Uta Jappe[4,5] and Katarzyna A. Duda[1]**

[1] Junior Research Group of Allergobiochemistry, Airway Research North (ARCN), German Center for Lung Research (DZL), Borstel, Germany, [2] Division of Experimental Pneumology, Research Center Borstel, Leibniz Lung Center, Airway Research Center North (ARCN), German Center for Lung Research (DZL), Borstel, Germany, [3] Institut des Biomolécules Max Mousseron, IBMM, UMR 5247, CNRS, ENSCM, University of Montpellier, Montpellier, France, [4] Division of Clinical and Molecular Allergology, Research Center Borstel, Leibniz Lung Center, Airway Research Center North (ARCN), German Center for Lung Research (DZL), Borstel, Germany, [5] Interdisciplinary Allergy Outpatient Clinic, Department of Pneumology, University of Lübeck, Lübeck, Germany

OPEN ACCESS

Edited by:
Uday Kishore, Brunel University London, United Kingdom

Reviewed by:
Lubna Kouser, Imperial College London, United Kingdom
Lakshna Mahajan, Swami Shraddhanand College, University of Delhi, India

***Correspondence:**
Katarzyna A. Duda
kduda@fz-borstel.de

Specialty section:
This article was submitted to Molecular Innate Immunity, a section of the journal Frontiers in Immunology

Received: *26 August 2018*
Accepted: *16 April 2019*
Published: *03 May 2019*

Citation:
González Roldán N, Engel R, Düpow S, Jakob K, Koops F, Orinska Z, Vigor C, Oger C, Galano J-M, Durand T, Jappe U and Duda KA (2019) Lipid Mediators From Timothy Grass Pollen Contribute to the Effector Phase of Allergy and Prime Dendritic Cells for Glycolipid Presentation. Front. Immunol. 10:974. doi: 10.3389/fimmu.2019.00974

Plant pollen are an important source of antigens that evoke allergic responses. Protein antigens have been the focus of studies aiming to elucidate the mechanisms responsible for allergic reactions to pollen. However, proteins are not the sole active agent present in pollen. It is known that pollen grains contain lipids essential for its reproduction and bioactive lipid mediators. These small molecular compounds are co-delivered with the allergens and hence have the potential to modulate the immune response of subjects by activating their innate immune cells. Previous reports showed that pollen associated lipid mediators exhibited neutrophil- and eosinophil-chemotactic activity and induced polarization of dendritic cells (DCs) toward a Th2-inducing phenotype. In our study we performed chemical analyses of the pollen associated lipids, that are rapidly released upon hydration. As main components we have identified different types of phytoprostanes (PhytoPs), and for the first time phytofurans (PhytoFs), with predominating 16-F_{1t}-PhytoPs (PPF_1-I), 9-F_{1t}-PhytoPs (PPF_1-II), 16-E_{1t}-PhytoPs (PPE_1-I) and 9-D_{1t}-PhytoPs (PPE_1-II), and 16(*RS*)-9-*epi*-ST-Δ^{14}-10-PhytoFs. Interestingly 16-E_{1t}-PhytoP and 9-D_{1t}-PhytoPs were found to be bound to glycerol. Lipid-containing samples (aqueous pollen extract, APE) induced murine mast cell chemotaxis and IL-6 release, and enhanced their IgE-dependent degranulation, demonstrating a role for these lipids in the immediate effector phase of allergic inflammation. Noteworthy, mast cell degranulation seems to be dependent on glycerol-bound, but not free phytoprostanes. On murine dendritic cells, APE selectively induced the upregulation of CD1d, likely preparing lipid-antigen presentation to iNKT cells. Our report contributes to the understanding of the activity of lipid mediators in the immediate effector phase of allergic reactions but identifies a yet undescribed pathway for the recognition of pollen-derived glycolipids by iNKT cells.

Keywords: pollen, timothy grass, phytoprostanes, phytofuranes, CD1d molecule, allergic airway inflammation

INTRODUCTION

Allergic diseases consist of several clinical conditions caused by hypersensitivity of the immune system to environmental factors that are normally harmless for most people. The prevalence of allergic diseases worldwide is rising dramatically in both developed and developing countries. This increase is especially problematic in children, who are bearing the greatest burden of the rising trend which has occurred over the last two decades (1). Grass pollen are, along with house dust mite, the most common inhalant allergen sources responsible for IgE-mediated allergies (2). An allergen is a protein, often glycosylated, that is presented by dendritic cells and together with additional factors leads to Th2 polarization and production of IgE antibodies (3). However, allergens are not delivered to the subject as pure proteins but as particles composed of various chemically different molecules in addition to the allergenic protein (4). The pollenkitt, an adhesive material present on the external layer of pollen grains, is mainly composed of lipids. These are responsible for reducing water loss and uptake, serve as reserve during pollen rehydration and activation of the stigma (5). Presently, the role of the lipids in the context of allergic inflammation is being increasingly discussed (6, 7). Lipids can be delivered alone, coming directly from the allergen source, as many different lipid species in different pollen samples with the ability to induce regulatory or stimulatory effects on the immune system, have been reported (8). They can either originate from microbes colonizing pollen (9) or be pollen-derived and transported bound to lipophilic allergens (6, 10).

Additionally, pollen grains contain lipid mediators that are released fast upon hydration, the so-called pollen associated lipid mediators (PALMs) (11). In the aqueous pollen extract (APE), containing PALMs hydroxy fatty acids-derivatives of linoleic acids (HODEs) (7) and phytoprostanes (PhytoPs) of class E_1-, F_1-, B_1-, and A_1 (12) were found. Birch and grass pollen activated and recruited polymorphonuclear granulocytes (PMNs) and eosinophils. This action was not fully mirrored by HODEs but rather by molecules structurally related to leukotrienes (LT). Cell activation was dependent on calcium mobilization and the upregulation of CD11b (7, 13). Additionally, PALMs were shown to elicit an effect on the activation and functional maturation of dendritic cells. In detail, birch pollen-APE selectively inhibited interleukin (IL)-12 p70 production of lipopolysaccharide (LPS)- or CD40L-activated DC, whereas IL-6, IL-10, and TNFα remained unchanged, leading to Th2 polarization. Interestingly this phenomenon was only dependent on PhytoP-E_1 and not PhytoP-F_1 or PhytoP-B_1 (12). In *in vivo* studies in a murine sensitization model, however, neither PhytoP-E_1 nor PhytoP-F_1 evoked Th2 polarization by DCs (14).

PhytoPs are products of non-enzymatic oxidation of α-linolenic acid, the most abundant polyunsaturated fatty acids in plants (15–17). They were first described in 1998 by Mueller and Parchmann (18) and are known to be biomarkers of oxidative stress in plants (18). Two nomenclatures have been published (19, 20). Mueller and co-workers used Rokach nomenclature and named the phytoprostanes PP type I and type II depending on the abstraction of hydrogen radical on α-linolenic acid. The Taber nomenclature was approved by IUPAC (International Union of Pure and Applied Chemistry) and will be further used in this manuscript. To avoid any confusion, Mueller nomenclature will be mentioned in brackets. Further, Jahn et al. published in 2010 a cautionary note on the correct assignment of all series of PhytoPs (21). In addition, due to isomeric complexity and chemical instability analytical characterization of PhytoPs is challenging.

Similarly to the recognition of microbial lipids, pollen-derived lipids was mediated by CD1-restricted T cells, namely invariant Natural Killer T (iNKT) cells (22). iNKT cells are a major population of innate-like T lymphocytes expressing a semi-invariant T cell receptor composed of a canonical Vα14-Jα18 α chain with a variable Vβ8, −7, or−2 β chain in mice or Vα24-Jα18/Vβ11 in humans that is specialized for recognition of glycolipid ligands (23). One of their most important features is the ability to rapidly release a broad spectrum cytokines (24), thus they can modulate various immune responses, which is dependent on the structure of the agonist. The role of iNKT cells in the context of allergy is still under debate. Scanlon et al. demonstrated the recruitment of iNKT cells to the airways in the presence of airborne lipid antigen (25), furthermore the requirement of iNKT cells for the initiation of airway inflammation has been demonstrated (26). In contrast, other reports have postulated only a modulatory role of iNKT cells in asthma (25). However, a crucial prerequisite for iNKT cell activation is the expression and modulation of the glycolipid-presenting molecule CD1d on antigen presenting cells, such as dendritic cells.

In this work, we aimed to characterize the chemical composition of the fast-released lipid fraction from Timothy grass pollen and to investigate their potential contribution to the effector phase of allergy. We also evaluated whether the fast-released lipids can modulate the recognition of grass pollen particle-bound glycolipids through the up-regulation of the glycolipid-presenting molecule CD1d.

MATERIALS AND METHODS

Preparation of Aqueous Pollen Extract (APE)

Pollen grains from Timothy grass (*Phleum pretense*) were purchased from Greer® and kept at −20°C prior use. Aqueous pollen extract (APE) was obtained according to Traidl-Hoffmann et al. (7), with some modifications. For chemical analyses, 44 mg of grass pollen were extracted with 1 ml of water (concentration of 44 mg/ml) and 30 mg per ml of PBS (for immunological assays) for 30 min in ultrasonic bath followed by centrifugation (3,900 × g, at 20°C). The concentration of 30 mg/ml was used as it has been reported that 34 mg/ml of APE resulted in an average concentration of 3.9×10^{10} mol/L LTB_4, which is known to be the concentration to induce migration in PMNs (7).

The supernatant containing fast-released lipids was sterile filtered (0.2 μm) and stored at −20°C prior to use.

In order to prepare protein free APE (APE_{ProtK}), APE was treated with Proteinase K (Roche) for 4 h at 56°C.

Enrichment and Fractionation of Lipids

To enrich fast-released lipids, PALMs, for a better detection in the analytical measurements, APE was further extracted with chloroform/methanol/water extraction (27) utilizing Branson Sonifier 250 for 20 min. on ice, Chloroform phase ($APE_{B/D}$) containing PALMs was sterile filtrated (0.2 μm), dried and further fractionated on the silica gel 60 column (10 × 1 cm; 0.04–0.063 mm, Merck) with increasing volumes of methanol. Fractions 3, 4, and 5 ($CHCl_3$/MeOH, 93/7, 90/10 and 80/20, v/v, respectively) were analyzed in detail utilizing GC/MS and LC/MS. Additionally, fraction 3 was further separated on reversed-phase Gilson 712 Gradient HPLC system equipped with Kromasil 100 C18, 5 μm, 250 × 10 mm (MZ-Analysentechnik GmbK) column with the following separation steps: isocratic 100% MeOH/H_2O (1/1, v/v), 30 min; isocratic 100% MeOH 30 min.; gradient 100% MeOH – 100% $CHCl_3$/MeOH (7/3, v/v) 30 min; isocratic 100% $CHCl_3$/MeOH (7/3, v/v), 30 min. Flow rate was 2 ml/min, and the eluting material was detected with a light scattering Sedex 55 detector (Sedere).

GC/MS Analyses

$APE_{B/D}$ and its fractions were analyzed in GC/MS after different derivatizations methods. Samples were either hydrolyzed with 2M HCl/MeOH (1 h, 85°C), followed by a peracetylation (10 min., 85°C) or with 2M NaOH, followed by a methylation with trimethylsilyldiazomethane (30 min., 22°C) and silylation with *N*,*O*-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) (3 h, 65°C). Additionally, to discriminate artifacts originating from acidic or alkaline hydrolysis $APE_{B/D}$ was directly methylated (omitting the hydrolysis step) with trimethylsilyldiazomethane (30 min., 22°C) and silylated with BSTFA (3 h, 65°C).

GC-MS measurements were performed on Agilent Technologies 7890A gas chromatograph equipped with a dimethylpolysiloxane column (Agilent, HP Ultra 1; 12 m × 0.2 mm × 0.33 μm film thickness) and 5975C series MSD detector with electron impact ionization (EI) mode under autotune condition at 70 eV. The temperature programme was 70°C for 1.5 min, then 60°C min^{-1} to 110°C and 5°C min^{-1} to 320°C for 10 min.

LC/MS Analyses

Phytoprostanes and phytofuranes profiling was performed using a micro-HPLC 200 plus (Eksingent Technologies, CA, USA) coupling with the tandem mass spectrometer Sciex QTRAP 5500 (Sciex Applied Biosystems, ON, Canada). Prior LC-MS injection, 100 mg of complete Timothy grass pollen was extracted with a Folch method according to the protocol of Yonny et al. (28) and Vigor et al. (29). The Timothy grass pollen extract (TGP), $APE_{B/D}$ and originating from $APE_{B/D}$ fractions 3, 4, and 5 underwent alkaline hydrolysis (1M KOH, 30 min., 40°C). Such obtained metabolites were concentrated by performing a solid phase extraction (SPE) step conducted on weak-anion exchange materials (Oasis MAX; 3 mL, 60 mg from Waters; Milford, MA, USA). Therewith metabolites were analyzed by micro-LC-MS/MS. The chromatographic separation of the phytoprostanoids was performed on a HALO C_{18} analytical column (100 × 0.5 mm, 2.7 μm particle size; Eksingent Technologies, CA, USA) held at 40°C and achieved by a gradient elution with 0.1% aqueous formic acid (A) and acetonitrile: methanol (80/20 v/v, B) with 0.1% additional formic acid. The gradient mode, delivered at 0.0 3mL.min-1, was started with 17% solvent B held for 1.6 min, increased to 21% solvent B at 2.85 min, to 25% at 7.27 min, to 28.4% at 8.8 min, to 33.1% at 9.62 min, to 33.3% at 10.95 min, and to 40% at 15 min. A maximum of 90% solvent B was reached at 16.47 min, and then returned to the initial conditions at 19 min. The Sciex QTRAP 5500 mass spectrometer detector operated in electrospray negative ionization mode. MS detection was performed by MS/MS using the MRM acquisition mode in a scheduled mode with an opening window of detection of ± 1 min (2 min in total) for the expected RT. Quantification of phytoprostanoids was achieved by the ratio between the peak area of each analyte and that of the corresponding IS. Data processing was achieved using MultiQuant 3.0 software (Sciex Applied Biosystems).

Mice

Cd1d$^{-/-}$ mice on a C57BL/6 background were kindly provided by Prof. Gisa Tiegs (UKE, Hamburg) and C57BL/6 controls were bred and housed at the Animal Care Facility of the Research Center Borstel. Mouse care and removal of organs was performed in accordance with institutional (RCB) guidelines.

ESI MS

Electrospray Ionization Mass Spectrometry (ESI MS) of $APE_{B/D\#3}$ was performed in negative ion mode using an amaZon speed ETD—Instrument (Bruker Daltonics) equipped with ESI ion source. Sample was dissolved at a concentration of ∼10 ng μL−1 in 10 mM ammonium acetate (50/50, v/v) mixture of chloroform and methanol and sprayed at a flow rate of 3 μL min−1. Capillary entrance voltage was set to 4.5 kV, and dry gas temperature to 180°C.

Degranulation of the Murine Mast Cells, Chemotaxis, and IL-6 Production

Bone marrow-derived mast cells (BMMCs) were generated by cultivation of bone marrow cells from C57BL/6 mice in the presence of recombinant murine IL-3 and stem cell factor (SCF). Cells were maintained in complete medium consisting of 10% heat-inactivated fetal calf serum (Biochrom), 50 μM β-mercaptoethanol, 1 mM sodium pyruvate, 2 mM L-glutamine, nonessential amino acids, penicillin, streptomycin (all from Gibco), and for the first 2 weeks 10 μg/mL ciprofloxacin (Bayer) or Myco-3 (Applichem A5240) in Iscove's modified Dulbecco medium (IMDM) (Gibco) supplemented with 10 ng/mL IL-3 and 10 ng/ml SCF (both from R&D). After 5 weeks of culture, >95% of the total cells were BMMCs ($CD117^+$ (c-Kit), $Fc\varepsilon RI\alpha^+$), $T1/ST2^+$ and were negative for mycoplasma contamination.

Chemotaxis of BMMCs was measured using a transwell chamber system by assessing migration through a polycarbonate filter insert of 8-μm pore size in 24-well-plates (Corning Life Sciences). BMMCs were preincubated with complete IMDM and 1 ng/ml IL-3 overnight. SCF (10 ng/ml) or APE (1:10 dilution from the original 30 mg/ml extraction of pollen) in assay buffer [IMDM with 5% BSA (Sigma)] were loaded into the lower

chambers in a volume of 600 μl. Cells were washed with assay buffer and added into the upper chamber (1×10^6 cells/ml in 100 μl) and incubated at 37°C and 5% CO_2 for 24 h. After incubation, cells from lower chambers were collected, washed in FACS-buffer (2% FCS, 0.1% NaN_3, 0.2 mM EDTA in PBS) with 2 μg/ml PI (Sigma) and the total cell number was determined by flow cytometry after addition of AccuCheck Counting beads (Invitrogen). Dead cells (PI^+) were excluded from analysis.

To induce degranulation or cytokine production 2×10^6 cells/ml were cultivated in the presence of 200 ng/mL of dinitrophenyl (DNP)–specific IgE (clone SPE-7) (Sigma) over night at 37°C and 5% CO_2. Cells were washed subsequently and stimulated either with PMA/Ionomycin (100 ng/mL, 100 nM, respectively), DNP-HSA as antigen (20 ng/mL, all from Sigma), APE, $APE_{B/D}$, 16-F_{1t}-PhytoP (PPF_1-I), 9-F_{1t}-PhytoP (PPF_1-II), or 9-D_{1t}-PhytoP (PPE_1-II) (20 μg/ml), in round bottom 96-well-plates for 20 min at 37°C for degranulation measurement. For IL-6 production cells were cultivated for 24 h and culture supernatants were collected. Degranulation was measured by the detection of the lysosomal membrane protein CD107a (LAMP-1) translocation to the cell surface. Cells were stained with anti-mouse CD107a (clone 1D4B), CD117 (clone 2B8) and FcεRIα (clone MAR-1) (all from BioLegend) and washed with FACS-buffer containing 2 μg/ml PI. PI-negative cells were analyzed by flow cytometry.

To analyze IL-6 production, supernatants from stimulated cells were collected and IL-6 concentration was measured by specific enzyme-linked immunosorbent assay (ELISA) using specific antibodies and standard protein from R&D Systems.

Maturation of Dendritic Cells

Bone marrow-derived dendritic cells (BMDCs) were generated by cultivation of bone marrow progenitor cells from C57BL/6 WT and *Cd1d*$^{-/-}$ mice in complete medium consisting of 10% heat-inactivated fetal calf serum (Biochrom), 1% L-glutamine, 1% penicillin, 1% streptomycin, 0.1% Mercaptoethanol (all from Gibco) in RPMI (Gibco) supplemented with 200 ng/ml GM-CSF (PeproTech). After 7 days of culture, BMDCs were harvested.

For stimulation BMDCs (2×10^5) from WT and *cd1d*$^{-/-}$ were cultivated in the presence of either APE (1:10 dilution originating from 30 mg/ml of pollen) or 100 ng/ml of highly purified and lipopeptide-free *S. friedenau* LPS (kindly provided by Prof. Helmut Brade, Research Center Borstel, Germany) in round bottom 96-well-plates for 24 h at 37°C in 5% CO_2. Maturation was assessed by the surface up-regulation of CD40, CD80, MHC class-II, and CD1d. Cells were labeled with anti-mouse CD80 (clone 16-10A1), CD1d (clone 1B1), CD40 (clone 3/23), CD11c (clone N418), MHC class-II (clone M5114.15.2) and washed in FACS-buffer containing 2 μg/ml PI. PI-negative cells were analyzed by flow cytometry.

Flow Cytometry

Samples for flow cytometry were acquired either on a FACScalibur, a LSR-II (BD Biosciences) or a MACSQuant 10 (Miltenyi Biotec). The generated data were analyzed using the FlowJo cell analysis software (FlowJo, LLC).

Statistical Analyses

Data are presented as mean values ± SD. Nonparametric one-way ANOVA and post tests were performed using GraphPad Prism version 5 software. A p-value of < 0.05 was considered as statistically significant.

RESULTS

Lipid Mediators of APE Are Phytoprostanes and Phytofuranes

APE was obtained from complete pollen grains of Timothy grass with the yield of 30%. Since such water extract contains apart of fast-released lipids also proteins, lipids were further enriched utilizing chloroform/methanol extraction, yielding $APE_{B/D}$ (0.44% to APE). Qualitative GC/MS analyses of $APE_{B/D}$ revealed the presence of different PhytoPs depending on the used derivatization protocol. In the samples after solvolysis (acidic hydrolysis) we have detected 16-A_{1t}-PhytoP (PPA_1-I) and 9-J_{1t}-PhytoP (PPA_1-II), after alkaline hydrolysis 16-B_{1t}-PhytoP (PPB_1-I) and 9-L_{1t}-PhytoP (PPB_1-II) and in preparations without any hydrolysis 16-E_{1t}-PhytoP (PPE_1-I) and 9-D_{1t}-PhytoP (PPE_1-II), not in a free form, but bound to glycerol (Gro). Since it is known that 16-E_{1t}-PhytoP (PPE_1-I) undergoes a dehydration to16-A_{1t}-PhytoP (PPA_1-I) and 16-B_{1t}-PhytoP (PPB_1-I); and 9-D_{1t}-PhytoP (PPE_1-II) to 9-J_{1t}-PhytoP (PPA_1-II) and 9-L_{1t}-PhytoP (PPB_1-II), we speculated that these species originated from16-E_{1t}-PhytoP (PPE_1-I) and 9-D_{1t}-PhytoP (PPE_1-II).

Fractionation on silica column yielded 7 fractions, but only in 3 of them, namely fraction 3, 4, and 5 we have detected lipid mediators. A major compound of fraction 3 analyzed after acidic hydrolysis was 16-A_{1t}-PhytoP (PPA_1-I) and 9-J_{1t}-PhytoP (PPA_1-II). After alkaline hydrolysis fraction 3 and 4 (0.028 and 0.048% to APE, respectively) contained 16-B_{1t}-PhytoP (**PPB_1**-I) and 9-L_{1t}-PhytoP (PPB_1-II), whereas fraction 5 (0.12% to APE) 16-F_{1t}-PhytoP (PPF_1-I) and 9-F_{1t}-PhytoP (PPF_1-II). Other detected compounds in various fractions were alkenes, alkanes, free fatty acids, di- and tri-hydroxy fatty acids.

Qualitative and quantitative LC/MS analysis of APE revealed the presence of PhytoPs from F_{1t}-, B_{1t}-, L_{1t}-, D_{1t}-series but also PhytoFs detected for the first time in pollen extracts, that would be considered to be new metabolites due to their recent discovery in nuts, seed, melon leaves or macroalgae (30). Importantly, the amounts of PhytoPs and PhytoFs detected in APE were comparable to those present in whole grass pollen (**Figure 1A**). We have enriched the lipid fraction of APE performing Bligh/Dyer ($APE_{B/D}$) extraction that led to the increase in detected lipids in factor of around 100 (**Figure 1B**). For a quantitative point of view, *ent*-16-B_{1t}-PhytoP (PPB_1-I) constituted the most abundant with a yield of 730 μg/g of pollen, while the lowest content was 55 μg/g of *ent*-16-F_{1t}-PhytoP (PPF_1-I). The content of PhytoFs reached values of 448, 155, and 90 μg/g for *ent*-16(*RS*)-9-*epi*-ST-Δ^{14}-10-PhytoF *ent*-16(*RS*)-13-epi-ST-Δ^{14}-9-PhytoF and *ent*-9(RS)-12-*epi*-ST-Δ^{10}-13-PhytoF, respectively. The separation of $APE_{B/D}$ on silica gel produced 3 fractions that presented specific profiles in terms of quality and quantity. Indeed, compared to $APE_{B/D}$ deprived of PhytoPs from

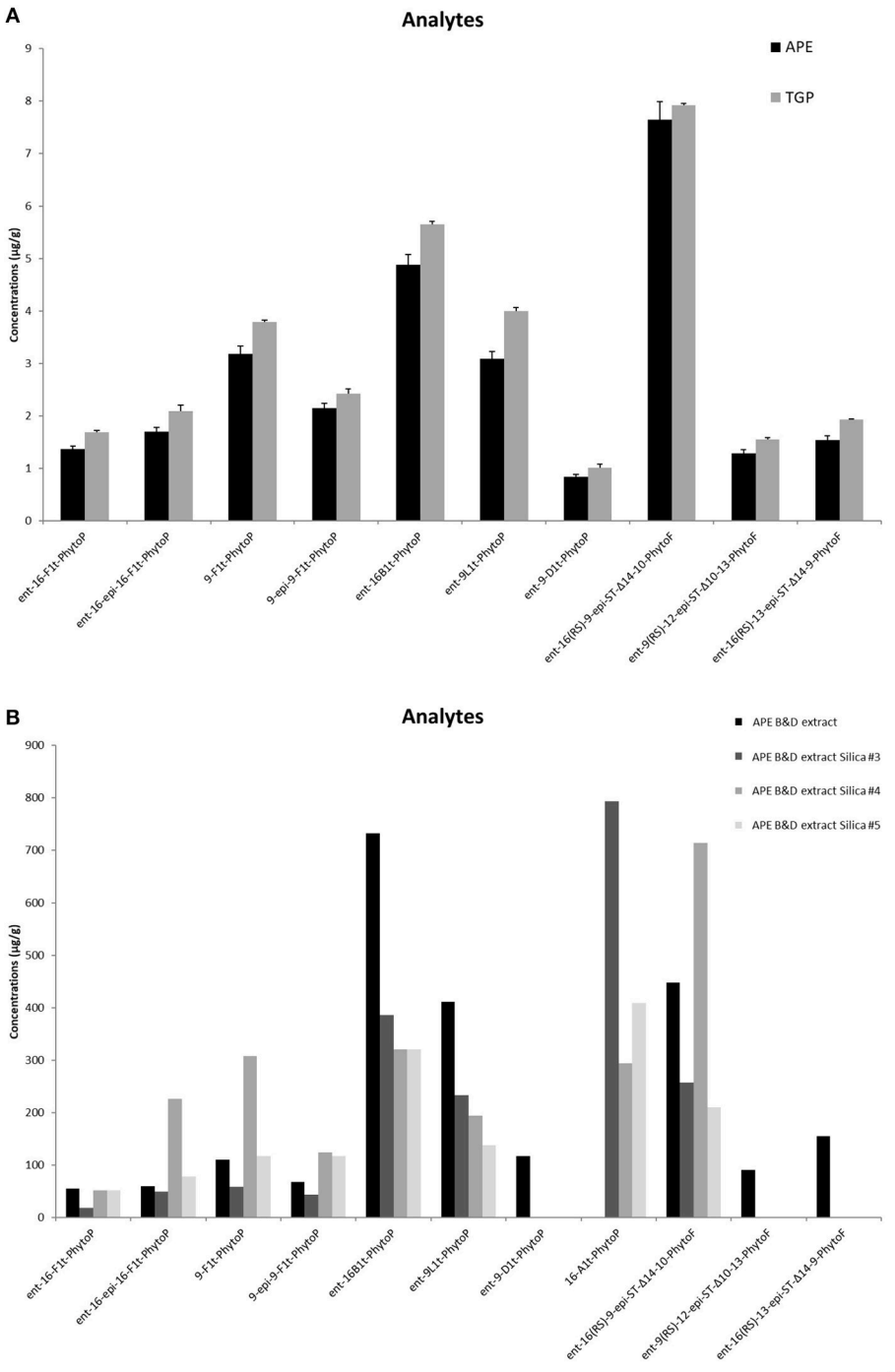


FIGURE 1 | Content of phytoprostanoids in Timothy grass pollen (TGP) and aqueous pollen extract (APE) **(A)**, and in the lipid-enriched $APE_{B/D}$ extract and fractions 3, 4, 5 obtained after silica gel fractionation of $APE_{B/D}$ **(B)** measured in LC/MS method. The content is given as mg/g of the sample. Quantification was achieved by the ratio between the peak area of each analyte and that of the corresponding internal standard.

A-serie, sub-fractions contained large amounts of 16-A_{1t}-phytoP, ranging from 293 to 793 µg/g. In contrast, *ent*-16(RS)-13-epi-ST-Δ^{14}-9-PhytoF and *ent*-9(RS)-12-*epi*-ST-Δ^{10}-13-PhytoF have been identified only in $APE_{B/D}$. If we compare the 3 sub-fractions to each other, the purification process allowed the concentration of 16-A_{1t}-PhytoP in fraction 3, while faction 4 concentrated in particular *ent*-16-*epi*-16-F_{1t}-phytoP, 9-F_{1t}-phytoP and *ent*-16(RS)-9-*epi*-ST-Δ^{14}-10-PhytoF.

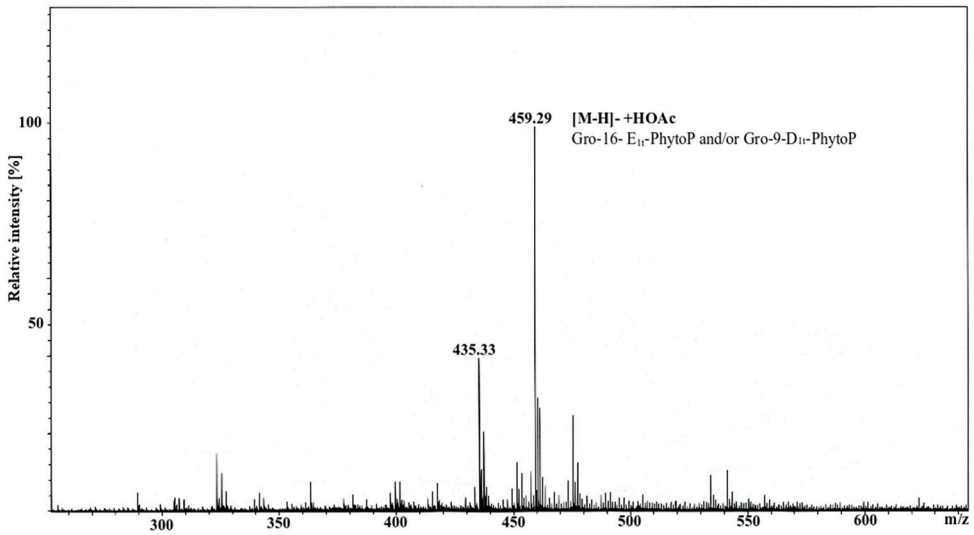


FIGURE 2 | ESI MS spectrum of fraction 3 originating from silica gel separation of $APE_{B/D}$. The measurement was performed in negative ion mode using an amaZon speed ETD. Molecular ion 459.29 (calculated 459.26) corresponded to $[M\text{-}H]^-$ +HOAc consisting of Gro-16E_{1t}-PhytoP/Gro-9-D_{1t}-PhytoP.

16-E_{1t}-PhytoP (PPE_1-I) and 9-D_{1t}-PhytoP (PPE_1-II) Are Bound to Glycerol

GC/MS analysis of $APE_{B/D}$ performed without any hydrolysis provided evidence that 16-E_{1t}-PhytoP (PPE_1-I) and 9-D_{1t}-PhytoP (PPE_1-II) are present not in a free form but bound to Gro. To further prove this, we run ESI MS analysis of fraction 3, and indeed the highest molecular ion 459.29 m/z corresponded to Gro-PhytoP-E_1 (**Figure 2**). As in GC/MS we have found both stereoisomers of PhytoP-E_1, namely 16-E_{1t}-PhytoP and 9-D_{1t}-PhytoP, both of them can be present in APE bound to Gro. We attempted to enrich this compound, and purified fraction 3 further on HPLC. This led to the isolation of Gro-16-E_{1t}-PhytoP and Gro-9-D_{1t}-PhytoP as determined by GC/MS (data not shown).

APE Recruits Mast Cells, Induces Their Activation, and Enhances Degranulation

Mast cells (MCs) are well-recognized as essential effector cells during allergic reactions, where their recruitment to the site of insult is critical (31). Hence, we were curious whether APE would induce chemotaxis of mast cells and influence their activation. BMMCs strongly migrated toward APE (**Figure 3A**), indicating the presence of chemotactic to mast cells compounds. This effect was even stronger than that of SCF, a known chemotactic factor for mast cells. MCs exert major effector functions by rapid degranulation and release of a wide range of mediators upon IgE-mediated FcεR crosslinking. BMMCs were treated with APE alone or during the induction of degranulation by sensitization with dinitrophenol (DNP)-specific IgE (IgE) followed by incubation with DNP-human albumin (Ag). Importantly, APE led to BMMCs degranulation in an IgE/Ag independent manner (**Figure 3B**) and significantly increased IgE-mediated degranulation of BMMCs. The same results we have obtained for protein-free APE (APE_{ProtK}) (not shown). Functionally, APE induced production of IL-6 (**Figure 3C**) in unsensitized BMMCs and enhanced IL-6 production in cells stimulated with IgE/Ag, overall indicating that APE has a capacity to recruit mast cells, activate them and induce their degranulation.

APE Selectively Induces CD1d Upregulation on Dendritic Cells

Expression of co-stimulatory molecules on dendritic cells influence further antigen recognition and polarization of T cells. To gain more understanding on the mechanisms how APE modulates immune responses and to evaluate whether APE could generally regulate recognition of other lipid classes such as glycolipids, we analyzed changes in the expression of CD1d on BMDCs in response to treatment with APE. *Cd1d*$^{-/-}$ BMDCs were used to rule out non-specific CD1d staining. Surface expression of CD1d on BMDC was significantly increased in APE stimulated BMDCs (**Figure 4A**). Contrary to LPS, known to induce expression of CD40, CD80, and MHC-class II, APE had no influence on the expression of these co-stimulatory molecules (**Figures 4B–D**).

Glycerol-Bound PhytoPs Mirror the Effect of APE on the BMMC Degranulation

Previous reports questioned the role of PhytoP in Th2 polarizing capacity of APE (14). Since our chemical analyses revealed that PhytoP in grass pollen are mainly present in a bound and not free form, we were prompted to test, whether the effect of APE on the IgE/Ag dependent degranulation of BMMCs would be reproduced by free or isolated by us fractions enriched with 16-E_{1t}-PhytoP and 9-D_{1t}-PhytoP. BMBCs were sensitized with IgE specific for DNP-human albumin (IgE) followed by

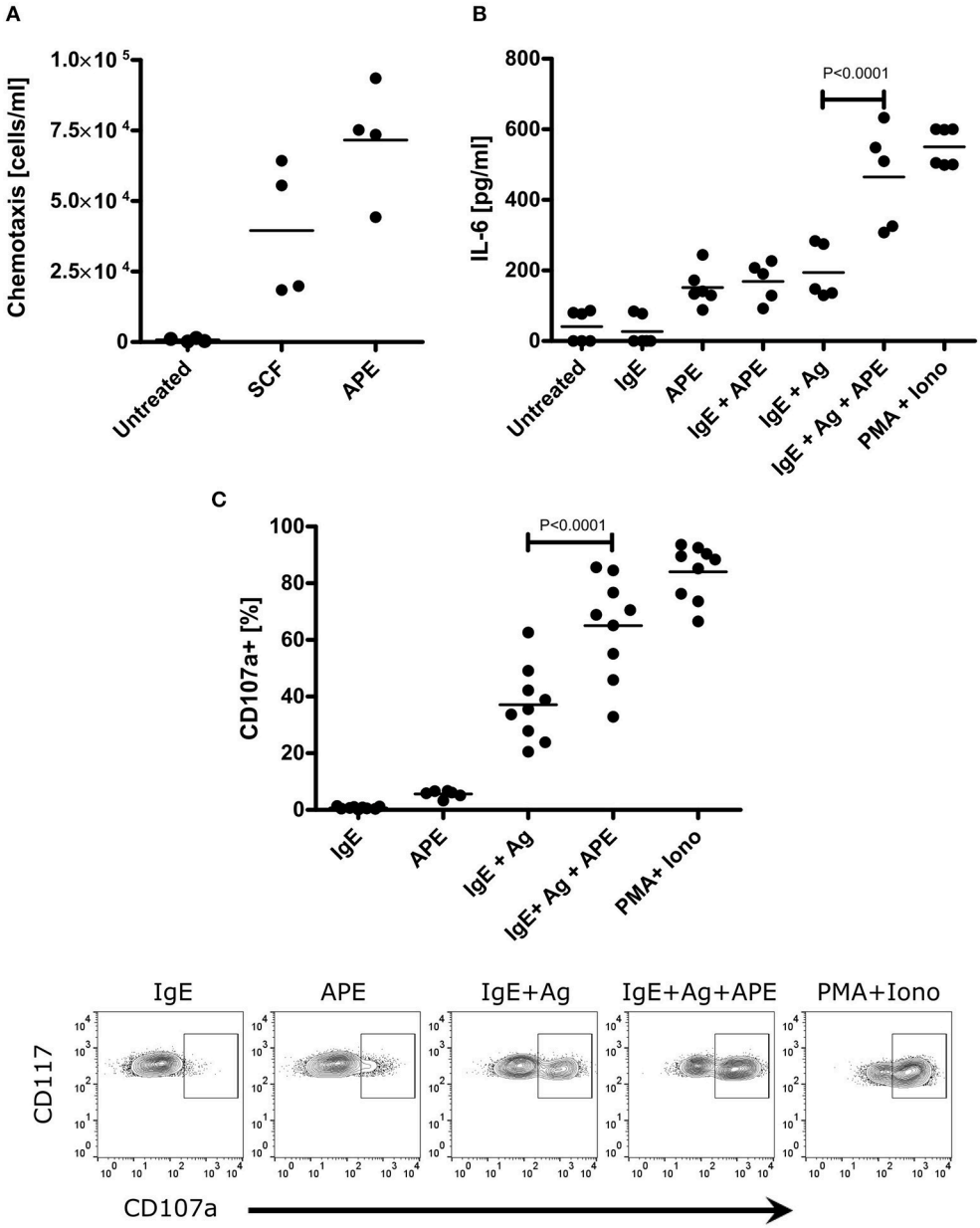


FIGURE 3 | APE induces BMMC chemotaxis, IL-6 production and enhances IgE/Ag-mediated effects. **(A)** BMMCs migrated through the polycarbonate filters toward APE. Assay buffer was used a negative control (marked as untreated), SCF was used as positive control. **(B)** APE induces IL-6 release in unsensitized BMMCs and enhances its production upon stimulation with IgE/Ag. BMMCs generated from C57BL6 mice were left untreated, stimulated with APE, DNP-HSA with or without IgE-sensitization. PMA and Ionomycin were used as positive control. IL-6 release into the supernatant was measured by ELISA. Data from two independent experiments ($n = 5$–6) are shown. **(C)** Incubation with APE induces BMMC degranulation (in average 6%) in IgE/Ag-independent manner and strongly enhance IgE/Ag-induced degranulation measured by CD107a translocation to plasma membrane. Incubation of BMMCs only in the presence of IgE was used as negative control and stimulation with PMA/Ionomycin was used as positive control. Cells were stained for CD117, FcεRIα and CD107a expression, and acquired on a BD FACScalibur flow cytometer. Bullet points represent the frequencies of FcεRIα$^+$CD117$^+$CD107a$^+$ (degranulated) BMMCs. Representative density plots (**C**, down) show how each of the treatments influence the degree of mast cell degranulation. Depicted are results out of 3 experiments generated from independent BMMC cultures ($n = 9$). Means ± SD are shown as lines. Statistical significance was calculated using the 1-way ANOVA analysis followed by Bonferroni's post-test for selected pairs of columns (IgE+Ag vs. IgE+Ag+APE).

incubation with DNP-human albumin (Ag) and co-incubated either with synthetic free PhytoP (16-F_{1t}-PhytoP (PPF_1-I), 9-F_{1t}-PhytoP (PPF_1-II), 9-D_{1t}-PhytoP (PPE_1-II), or APE, $APE_{B/D}$, $APE_{B/D\#3}$ (possessing as major compounds 16-E_{1t}-PhytoP, PPE_1-I and 9-D_{1t}-PhytoP, PPE_1-II). Importantly, only APE, $APE_{B/D}$, $APE_{B/D\#3}$ and not free PhytoP led to increased IgE/Ag dependent

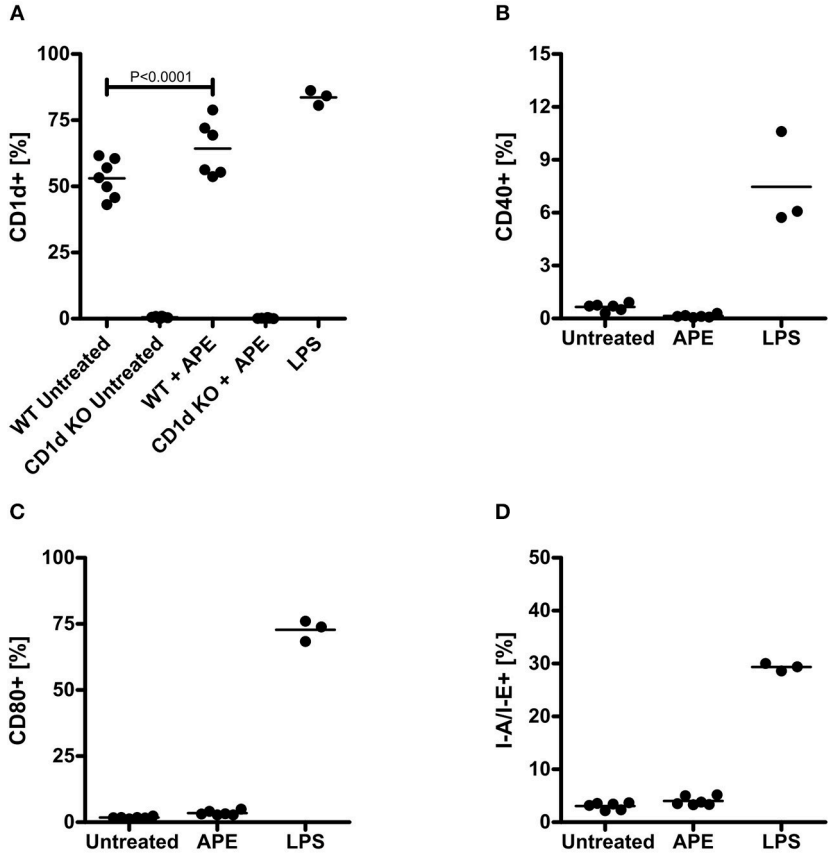


FIGURE 4 | Activation of dendritic cells by APE. APE selectively induced expression of CD1d **(A)** but not of CD40, CD80 and MHC class II on BMDCs **(B–D)**. Negative control BMDCs were left unstimulated (marked as untreated) and positive control BMMC were activated with LPS. Cells were stained for CD1d, CD40, CD80, and MHC-class II expression and analyzed on a BD FACScalibur flow cytometer. Bullet points represent the percentage of cells expressing the corresponding activation markers. Graph shows combined results out of 2 experiments generated from independent BMDC cultures ($n = 3$–7). Means ± SD are shown as lines. Statistical significance was calculated using the 1-way ANOVA analysis followed by Bonferroni's Multiple Comparison post-test.

degranulation of BMMCs, indicating that biological activity of APE might be mediated by bound-PhytoP (**Figure 5**). The effect observed for $APE_{B/D}$ was statistically significant.

DISCUSSION

It has been demonstrated that pollen in addition to liberating protein allergens rapidly release various bioactive lipids into the aqueous phase (32, 33). These pollen-associated lipid mediators (PALMs) were shown to stimulate and recruit cells of the innate immune system, such as neutrophils (7) and eosinophils (13). Furthermore, PALMs influenced the activation and functional maturation of human DCs toward a Th2 type (12). APE, the protein-free fraction of ragweed pollen extracts (Amb-APE), and the pollen-derived PhytoP-E_1 were shown to be responsible for B-cell-dependent aggravation of IgE-mediated allergies (34). In this report the stereoisomer of PhytoP-E_1 was not further specified.

LC/MS analysis of APE and $APE_{B/D}$ revealed that the content of phytoprostanoids (μg/g) was enriched in $APE_{B/D}$ by a factor of 100. In comparison with the previous reports (12) we have determined specifically the stereoisomers of PhytoP found in grass pollen, e.g., 16-E_{1t}-PhytoP and to our knowledge for the first time reported the presence of PhytoF. PhytoFs are tetrahydrofuran ring containing compounds created as PhytoP from α-linolenic acid (ALA,18:3 n-3) and are proposed to be indicators of oxidative stress in plants, similarly to PhytoPs (30). Since the content of PhytoF was even higher than those of PhytoP it would be interesting to evaluate the role of synthetic PhytoF in the context of allergy.

Our analyses did not show any presence of hydroxy fatty acids-derivatives of linoleic acids (HODEs) in Timothy grass pollen contrary to work of Traidl-Hoffmann et al. (7). Along with the work of Imbusch and Mueller indicating that levels of esterified PhytoP-F_1 and PhytoP-E_1 were one to two orders of magnitude higher than those of free PhytoPs (35) we have found that 16-E_{1t}-PhytoP (PPE_1-I) and 9-D_{1t}-PhytoP (PPE_1-II) were bound to glycerol. Since we did not detect any 16-F_{1t}-PhytoP (PPF_1-I) and 9-F_{1t}-PhytoP (PPF_1-II) in the sample without hydrolysis, and in the samples after hydrolysis first after a fractionation step, we speculate that these PhytoP species are also esterified, having a molecular weight too high for the limit detection in GC/MS.

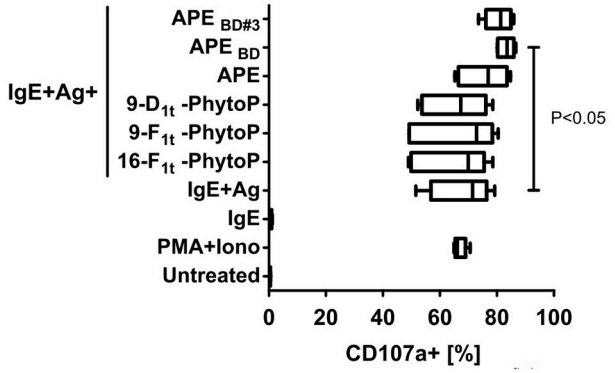


FIGURE 5 | Glycerol-bound phytoprostanes are responsible for the enhancement of MC degranulation. $APE_{B/D}$ (containing enriched Gro-phytoprostanes) and enriched Gro-16E_{1t}-PhytoP/Gro-9-D_{1t}-PhytoP ($APE_{B/D\#3}$) fraction but not free PhytoP led to enhanced IgE/Ag-induced degranulation of BMMCs as measured by CD107a translocation to plasma membrane. Negative control BMMCs were left unstimulated (marked as untreated) and positive control BMMC were activated with PMA and Ionomycin. Cells were stained for CD117 and CD107a and acquired on a MACSQuant10 flow cytometer. Box & whiskers plots represent the frequencies of degranulated ($CD107a^+$) BMMCs ($CD117^+$ events). Graph shows combined results out of 2 experiments generated from independent BMMC cultures ($n = 6$). Means ± SD are shown as lines. Statistical significance was calculated by One-way ANOVA followed by Dunnett's multiple comparison post-test to a control group (IgE+Ag).

Definitively the method of choice for the detailed qualitative and quantitative analyses of prostanoids is LC/MS. This is a very sensitive method and due to application of internal standards (synthetic neuroprostane (36)) the identification of different isomers is achieved. The limitation of LC/MS is the detection of artifacts originating from products of alkaline hydrolysis and incapability of measuring bound (esterified) molecules.

The disadvantage of GC/MS is the requirement of a derivatizing process and the inability to separate isomeric compounds with the same molecular weight. Fortunately, we have succeeded in performing GC/MS analyses on the sample without hydrolysis and by doing so detected 16-E_{1t}-PhytoP (PPE_1-I) and 9-D_{1t}-PhytoP (PPE_1-II) bound to glycerol. This finding we have reproduced in ESI MS experiment of fraction 3. Thus, the combination of the analytical protocols is of great value to understand the chemical content of biological samples. The fact that 16-E_{1t}-PhytoP (PPE_1-I) and 9-D_{1t}-PhytoP (PPE_1-II) (we speculate that this is also the case for 16-F_{1t}-PhytoP (PPF_1-I) and 9-F_{1t}-PhytoP (PPF_1-II)) is not present in free but bound form, may explain why E_1- and F_1-PhytoPs did not display Th2-polarizing capacities *in vivo* (14) as has been shown in *in vitro* studies (12). Our results showing that APE, $APE_{B/D}$, $APE_{B/D\#3}$ and not free PhytoP led to an increased IgE/Ag dependent degranulation of BMMCs support this hypothesis. It is also tempting to speculate that PhytoFs, found for the first time in grass pollen may be another important player in APE.

Mast cells are strategically located at sites that are continuously exposed to the environment, such as mucosal surfaces, and together with their immediate effector activity, they are considered as sentinels of the immune system. In line with this concept, the notion that lipid mediators are immediately released upon contact with the mucosal surfaces, they potentially recruit or induce an accumulation of mast cells to the site of release as evidenced by their recruitment by PALMs. Mast cells are thus the third type of effector cells of allergic reaction, along with neutrophils and eosinophils to which PALMs of pollen grass act chemotactic (7, 13).

In addition, dendritic cells would be in turn also recruited and activated by PALMs. During the process of maturation, dendritic cells migrate to the regional lymph node and prepare for antigen presentation. Interestingly, PALMs induced a selective upregulation of CD1d, probably by activation through PPARγ ligation (37, 38), which could indicate that PALMs induces a maturation program that prepares DCs for glycolipid presentation of potential glycolipid ligands co-delivered with PALMs to mucosal surfaces. Further analyses are required to assess the relevance and significance of this pathway for the process of allergic reactions.

This is the first report evidencing PALMs induced upregulation of CD1d. The work of Abós-Gracia et al. (38) demonstrated that the exposure to total lipid extract but not APE of olive pollen to DCs upregulated Cd1d expression that led to the activation of invariant Natural Killer cells (iNKT). Interestingly, this phenomenon was in accordance with our results not linked to the increased expression of other maturation markers, such as CD80 and CD86.

In summary, our work clearly presented that the chemical composition of fast-released lipids in grass pollen is much more complex than evidenced before. Moreover, the function of molecules originating from heterogenous biological systems, such as sources of allergens, has to be synchronized with the knowledge of detailed chemical structure. Functionally, with our work and others, it became clear that PALMs contribute to the initiation and effector phase of allergy, having the capacity to attract/activate major players of allergic inflammation (neutrophils, eosinophils, mast cells). We have additionally evidenced that PALMs may prime the immune system for further recognition of glycolipids by NKT cells through the promotion of the expression of CD1d molecules.

ETHICS STATEMENT

Mouse care and removal of organs was performed in accordance with institutional (Research Center Borstel) guidelines. The full ethics comittee approval was not required, according to the local and national guidelines.

AUTHOR CONTRIBUTIONS

NG and KD contributed to the conception and design of the study and interpretation of the data. NG performed dendritic maturation test and the statistical analysis. SD performed mast cell degranulation test. ZO and FK performed mast cell degranulation test, chemotaxis and measured IL-6 production. RE prepared and fractionated

APE and $APE_{B/D}$ extracts, performed GC/MS analyses. KJ performed ESI MS analyses. J-MG and TD prepared synthetic phytoprostanes. CV and CO performed LC/MS analyses. KD wrote the first draft of the manuscript. UJ contributed to the interpretation of the data and critically revised the manuscript. NG, KD, and CV prepared figures. KD, NG, CV, and TD wrote sections of the manuscript. All authors contributed to the manuscript revision, read and approved the submitted version.

FUNDING

This work was supported by VIP Funds of Research Center Borstel (to NG and KD).

ACKNOWLEDGMENTS

The authors thank the Fluorescence Cytometry Core facility at RCB.

REFERENCES

1. Devereux G. The increase in the prevalence of asthma and allergy: food for thought. *Nat Rev Immunol.* (2006) 6:869–74. doi: 10.1038/nri1958
2. Worm M, Lee H-H, Kostev K. Prevalence and treatment profile of patients with grass pollen and house dust mite allergy: allergic rhinitis - incidence and treatment. *J Deutschen Dermatolog Gesellschaft*. (2013) 11:653–61. doi: 10.1111/ddg.12089
3. Lambrecht BN, Hammad H. The immunology of asthma. *Nat Immunol.* (2015) 16:45–56. doi: 10.1038/ni.3049
4. Woodfolk JA, Commins SP, Schuyler AJ, Erwin EA, Platts-Mills TAE. Allergens, sources, particles, and molecules: why do we make IgE responses? *Allergol. Int.* (2015) 64:295–303. doi: 10.1016/j.alit.2015.06.001
5. Pacini E, Hesse M. Pollenkitt – its composition, forms and functions. *Flora Morphol Distribution Funct Ecol Plants*. (2005) 200:399–415. doi: 10.1016/j.flora.2005.02.006
6. Bublin M, Eiwegger T, Breiteneder H. Do lipids influence the allergic sensitization process? *J Allergy Clin Immunol.* (2014) 134:521–9. doi: 10.1016/j.jaci.2014.04.015
7. Traidl-Hoffmann C, Kasche A, Jakob T, Huger M, Plötz S, Feussner I, et al. Lipid mediators from pollen act as chemoattractants and activators of polymorphonuclear granulocytes. *J Allergy Clin Immunol.* (2002) 109:831–8. doi: 10.1067/mai.2002.124655
8. Bashir MEH, Lui JH, Palnivelu R, Naclerio RM, Preuss D. Pollen Lipidomics: lipid profiling exposes a notable diversity in 22 allergenic pollen and potential biomarkers of the allergic immune response. *PLoS ONE*. (2013) 8:e57566. doi: 10.1371/journal.pone.0057566
9. Heydenreich B, Bellinghausen I, König B, Becker W-M, Grabbe S, Petersen A, et al. Gram-positive bacteria on grass pollen exhibit adjuvant activity inducing inflammatory T cell responses. *Clin Exp Allergy*. (2012) 42:76–84. doi: 10.1111/j.1365-2222.2011.03888.x
10. Mogensen JE, Wimmer R, Larsen JN, Spangfort MD, Otzen DE. The major birch allergen, Bet v 1, shows affinity for a broad spectrum of physiological ligands. *J Biol Chem.* (2002) 277:23684–92. doi: 10.1074/jbc.M202065200
11. Gilles S, Mariani V, Bryce M, Mueller MJ, Ring J, Behrendt H, et al. Pollen allergens do not come alone: pollen associated lipid mediators (PALMS) shift the human immue systems towards a TH2-dominated response. *Allergy Asthma Clin Immunol.* (2009) 5:3. doi: 10.1186/1710-1492-5-3
12. Traidl-Hoffmann C, Mariani V, Hochrein H, Karg K, Wagner H, Ring J, et al. Pollen-associated phytoprostanes inhibit dendritic cell interleukin-12 production and augment T helper type 2 cell polarization. *J Exp Med.* (2005) 201:627–36. doi: 10.1084/jem.20041065
13. Plötz SG, Traidl-Hoffmann C, Feussner I, Kasche A, Feser A, Ring J, et al. Chemotaxis and activation of human peripheral blood eosinophils induced by pollen-associated lipid mediators. *J Allergy Clin Immunol.* (2004) 113:1152–60. doi: 10.1016/j.jaci.2004.03.011
14. Gutermuth J, Bewersdorff M, Traidl-Hoffmann C, Ring J, Mueller MJ, Behrendt H, et al. Immunomodulatory effects of aqueous birch pollen extracts and phytoprostanes on primary immune responses *in vivo*. *J Allergy Clin Immunol.* (2007) 120:293–9. doi: 10.1016/j.jaci.2007.03.017
15. Durand T, Bultel-Poncé V, Guy A, El Fangour S, Rossi J-C, Galano J-M. Isoprostanes and phytoprostanes: bioactive lipids. *Biochimie*. (2011) 93:52–60. doi: 10.1016/j.biochi.2010.05.014
16. Durand T, Bultel-Poncé V, Guy A, Berger S, Mueller MJ, Galano J-M. New bioactive oxylipins formed by non-enzymatic free-radical-catalyzed pathways: the phytoprostanes. *Lipids*. (2009) 44:875–88. doi: 10.1007/s11745-009-3351-1
17. Galano J-M, Lee JC-Y, Gladine C, Comte B, Le Guennec J-Y, Oger C, et al. Non-enzymatic cyclic oxygenated metabolites of adrenic, docosahexaenoic, eicosapentaenoic and α-linolenic acids; bioactivities and potential use as biomarkers. *Biochim Biophys Acta Mol Cell Biol Lipids*. (2015) 1851:446–55. doi: 10.1016/j.bbalip.2014.11.004
18. Parchmann S, Mueller MJ. Evidence for the formation of dinor isoprostanes E_1 from α-linolenic acid in plants. *J Biol Chem.* (1998) 273:32650–5. doi: 10.1074/jbc.273.49.32650
19. Taber DF, Morrow JD, Jackson Roberts L. A nomenclature system for the isoprostanes. *Prostaglandins*. (1997) 53:63–7. doi: 10.1016/S0090-6980(97)00005-1
20. Rokach J, Khanapure S, Hwang S-W, Adiyaman M, Lawson J, FitzGerald G. Nomenclature of Isoprostanes: a proposal. *Prostaglandins*. (1997) 54:853–73. doi: 10.1016/S0090-6980(97)00184-6
21. Jahn U, Galano J-M, Durand T. A cautionary note on the correct structure assignment of phytoprostanes and the emergence of a new prostane ring system. *Prostaglandins Leukotrienes Essential Fatty Acids*. (2010) 82:83–6. doi: 10.1016/j.plefa.2009.10.005
22. Agea E, Russano A, Bistoni O, Mannucci R, Nicoletti I, Corazzi L, et al. Human CD1-restricted T cell recognition of lipids from pollens. *J Exp Med.* (2005) 202:295–308. doi: 10.1084/jem.20050773
23. Bendelac A, Savage PB, Teyton L. The biology of NKT cells. *Ann Rev Immunol.* (2007) 25:297–336. doi: 10.1146/annurev.immunol.25.022106.141711
24. Matsuda JL, Mallevaey T, Scott-Browne J, Gapin L. CD1d-restricted iNKT cells, the 'Swiss-Army knife' of the immune system. *Curr Opin Immunol.* (2008) 20:358–68. doi: 10.1016/j.coi.2008.03.018
25. Scanlon ST, Thomas SY, Ferreira CM, Bai L, Krausz T, Savage PB, et al. Airborne lipid antigens mobilize resident intravascular NKT cells to induce allergic airway inflammation. *J Exp Med.* (2011) 208:2113–24. doi: 10.1084/jem.20110522
26. Wingender G, Rogers P, Batzer G, Lee MS, Bai D, Pei B, et al. Invariant NKT cells are required for airway inflammation induced by environmental antigens. *J Exp Med.* (2011) 208:1151–62. doi: 10.1084/jem.20102229
27. Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. *Can J Biochem Physiol.* (1959) 37:7.
28. Yonny ME, Rodríguez Torresi A, Cuyamendous C, Réversat G, Oger C, Galano J-M, et al. Thermal stress in melon plants: phytoprostanes and phytofurans as oxidative stress biomarkers and the effect of antioxidant supplementation. *J Agric Food Chem.* (2016) 64:8296–304. doi: 10.1021/acs.jafc.6b03011
29. Vigor C, Reversat G, Rocher A, Oger C, Galano J-M, Vercauteren J, et al. Isoprostanoids quantitative profiling of marine red and brown macroalgae. *Food Chem.* (2018) 268:452–62. doi: 10.1016/j.foodchem.2018.06.111
30. Cuyamendous C, de la Torre A, Lee YY, Leung KS, Guy A, Bultel-Poncé V, et al. The novelty of phytofurans, isofurans, dihomo-isofurans and neurofurans: discovery, synthesis and potential application. *Biochimie*. (2016) 130:49–62. doi: 10.1016/j.biochi.2016.08.002
31. Williams CMM, Galli SJ. The diverse potential effector and immunoregulatory roles of mast cells in allergic disease. *J Allergy Clin Immunol.* (2000) 105:847–59. doi: 10.1067/mai.2000.106485

32. Imbusch R, Mueller MJ. Formation of isoprostane F(2)-like compounds (phytoprostanes F(1)) from alpha-linolenic acid in plants. *Free Radic Biol Med*. (2000) 28:720–6. doi: 10.1016/S0891-5849(00)00154-4
33. Mueller MJ. Archetype signals in plants: the phytoprostanes. *Curr Opin Plant Biol*. (2004) 7:441–8. doi: 10.1016/j.pbi.2004.04.001
34. Oeder S, Alessandrini F, Wirz OF, Braun A, Wimmer M, Frank U, et al. Pollen-derived nonallergenic substances enhance Th2-induced IgE production in B cells. *Allergy*. (2015) 70:1450–60. doi: 10.1111/all.12707
35. Imbusch R, Mueller MJ. Analysis of oxidative stress and wound-inducible dinor isoprostanes F_1 (Phytoprostanes F_1) in plants. *Plant Physiol.* (2000) 124:1293–304. doi: 10.1104/pp.124.3.1293
36. Oger C, Bultel-Poncé V, Guy A, Balas L, Rossi J-C, Durand T, et al. The handy use of brown's P2-Ni catalyst for a skipped diyne deuteration: application to the synthesis of a [D4]-labeled F4t-neuroprostane. *Chem A Eur J.* (2010) 16:13976–80. doi: 10.1002/chem.201002304
37. Varga T, Czimmerer Z, Nagy L. PPARs are a unique set of fatty acid regulated transcription factors controlling both lipidmetabolism and inflammation. *Biochim Biophys Acta Mol Basis Dis.* (2011) 1812:1007–22. doi: 10.1016/j.bbadis.2011.02.014
38. Abós-Gracia B, del Moral MG, López-Relaño J, Viana-Huete V, Castro L, Villalba M, et al. Olea europaea pollen lipids activate invariant natural killer T cells by upregulating CD1d expression on dendritic cells. *J Allergy Clin Immunol.* (2013) 131:1393–9.e5. doi: 10.1016/j.jaci.2012.11.014

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

MINI REVIEW
published: 29 May 2019
doi: 10.3389/fimmu.2019.01174

Modulation of Mast Cell Reactivity by Lipids: The Neglected Side of Allergic Diseases

*Philipp M. Hagemann[1], Stephanie Nsiah-Dosu[2], Jennifer Elisabeth Hundt[3], Karin Hartmann[2,4] and Zane Orinska[1]**

[1] Division of Experimental Pneumology, Research Center Borstel, Leibniz Lungenzentrum, Airway Research Center North, German Center for Lung Research (DZL), Borstel, Germany, [2] Department of Dermatology, University of Luebeck, Luebeck, Germany, [3] Luebeck Institute of Experimental Dermatology, University of Luebeck, Luebeck, Germany, [4] Division of Allergy, Department of Dermatology, University of Basel, Basel, Switzerland

Mast cells (MCs) have long been mainly regarded as effector cells in IgE-associated allergic disorders with potential immunoregulatory roles. Located close to the allergen entry sites in the skin and mucosa, MCs can capture foreign substances such as allergens, toxins, or noxious substances and are exposed to the danger signals produced by epithelial cells. MC reactivity shaped by tissue-specific factors is crucial for allergic responses ranging from local skin reactions to anaphylactic shock. Development of Th2 response leading to allergen-specific IgE production is a prerequisite for MC sensitization and induction of FcεRI-mediated MC degranulation. Up to now, IgE production has been mainly associated with proteins, whereas lipids present in plant pollen grains, mite fecal particles, insect venoms, or food have been largely overlooked regarding their immunostimulatory and immunomodulatory properties. Recent studies, however, have now demonstrated that lipids affect the sensitization process by modulating innate immune responses of epithelial cells, dendritic cells, and NK-T cells and thus crucially contribute to the outcome of sensitization. Whether and how lipids affect also MC effector functions in allergic reactions has not yet been fully clarified. Here, we discuss how lipids can affect MC responses in the context of allergic inflammation. Direct effects of immunomodulatory lipids on MC degranulation, changes in local lipid composition induced by allergens themselves and changes in lipid transport affecting MC reactivity are possible mechanisms by which the function of MC might be modulated.

Keywords: mast cells, degranulation, allergy, lipids, lipid mediators, flippases, floppases, scramblases

OPEN ACCESS

Edited by:
Otto Holst, Forschungszentrum Borstel (LG), Germany

Reviewed by:
Valerio Chiurchiù, Campus Bio-Medico University, Italy
Ivana Halova, Institute of Molecular Genetics (ASCR), Czechia

***Correspondence:**
Zane Orinska
zorinska@fz-borstel.de

Specialty section:
This article was submitted to Inflammation, a section of the journal Frontiers in Immunology

Received: *22 September 2018*
Accepted: *08 May 2019*
Published: *29 May 2019*

Citation:
Hagemann PM, Nsiah-Dosu S, Hundt JE, Hartmann K and Orinska Z (2019) Modulation of Mast Cell Reactivity by Lipids: The Neglected Side of Allergic Diseases. Front. Immunol. 10:1174. doi: 10.3389/fimmu.2019.01174

INTRODUCTION

Mast cells are long-living tissue-resident hematopoietic cells equipped with secretory granules containing a broad spectrum of biologically active mediators such as histamine, proteases, and cytokines (1, 2). Preferentially located in the skin and mucosa, MCs detect potentially dangerous or noxious substances in concert with danger signals produced by epithelial cells at damaged barriers. Extensive MC degranulation as an urgent response to different types of stimulation and its wide-ranging local or systemic effects are the reasons why MCs are the main effector cells in allergies (3). Here, we summarize recent findings describing how reactivity of MCs can be modulated by lipids and discuss how interference with intracellular lipid transport could affect MC reactivity.

LIPID STRUCTURE AND MOLECULAR FEATURES

Lipids are overall hydrophobic or amphipathic molecules consisting of a hydrophilic head group and a hydrophobic tail group connected either by esters or ether bonds. Other lipids like sterols consist of a ring structure with various modifications. Lipids, in contrast to proteins and nucleic acids, are synthesized by a series of specific interlinked enzymatic reactions generating a high diversity of different lipid molecules. According to their hydrophobic characteristics and chemically functional backbones, lipids are categorized into eight main groups, namely fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, sterol lipids, prenol lipids, saccharolipids, and polyketides (http://www.lipidmaps.org). Lipids are essential in storage of energy, arrangement of signaling complexes, participation in signal processing as second messengers and building of membranes as physical barriers. Membranes in mammalian cells consist mainly of sphingolipids, glycerophospholipids, and cholesterol (4). They are fluidic bilayers characterized by different lipid compositions in their inner and outer sides where lipids together with proteins form highly ordered structures essential for organization of cellular compartments. An integral part of membranes is cholesterol. It is synthesized in the endoplasmic reticulum (ER), transported then to the Golgi complex, and further to the plasma membrane which shows the highest cholesterol concentration. Together with sphingolipids, cholesterol regulates the membrane permeability and facilitates organization of ordered protein islets. Glycerophospholipids and sphingolipids are also synthesized in the ER and further modified in the Golgi complex as well as the mitochondria (5–7). The lysosome, on the other hand, plays a crucial role in lipid sorting and metabolism (8). Enzymatically induced changes in the lipid composition of the membrane are associated with a new ordering of membrane proteins and altered membrane microdomains (9). In general, any change in the lipid or protein compartment of a membrane affects both partners and is therefore tightly controlled by the cell. MCs in particular undergo dramatic membrane reorganization while degranulation and recovery. The schematic structure of the different lipid categories and exemplary representatives of these categories with effects on MC functions are summarized in **Table 1**.

PRODUCTION OF LIPID MEDIATORS IS ASSOCIATED WITH CHANGES IN MAST CELL REACTIVITY

Activated in settings of allergic responses, mainly through stimulation of the high affinity receptor FcεRI complex by IgE-recognizing specific antigens, MCs release pre-stored biogenic amines, proteases, proteoglycans, chemokines as well as cytokines. In addition, MCs are well-known producers of different lipid mediators such as leukotrienes (LT) and prostaglandins (PG) (14, 20, 35) and production of these lipid mediators has in turn been shown to regulate MC functions. For example, MC-produced lipid mediators have been found to enhance inflammation in specific situations (36, 37) or to limit inflammation in other circumstances associated with reestablishment of tissue homeostasis (38, 39). Recent studies also demonstrated that enzymes responsible for production of lipid mediators belong to the MC-specific gene expression signature (40). Furthermore, *in vitro* generated connective tissue-like MCs and mucosal-like MCs differ in their eicosanoid patterns (41) and skin MCs are unique in showing the lowest expression levels of *Alox5* gene encoding 5-lipoxygenase (40), indicating that lipid mediator production is coordinated by tissue-specific regulatory mechanisms. Production of eicosanoid mediators, sphingolipid metabolites, and platelet-activating factor (PAF) by MCs is extensively reviewed elsewhere (15, 42). Thus, during allergic responses, MCs produce a variety of lipid mediators acting in a paracrine and autocrine manner. In addition, MC reactivity is modulated by lipid mediators produced by other cells exposed to environmental challenges.

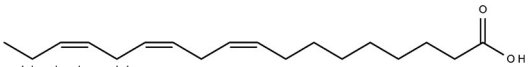
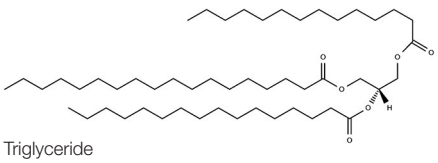
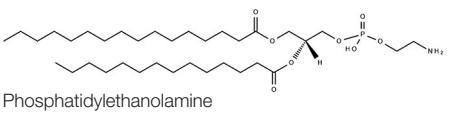
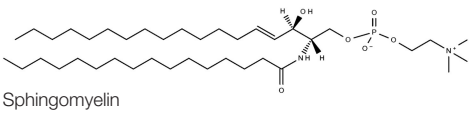
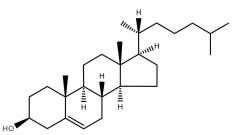
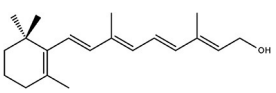
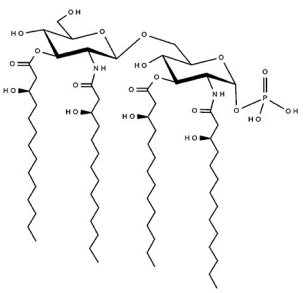
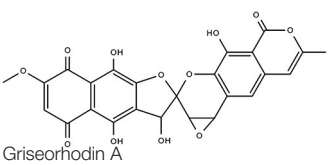
ENDOCANNABINOIDS AFFECT MAST CELL REACTIVITY

Often overlooked regarding their modulatory effects on MC function are endocannabinoids-a group of bioactive lipids serving as secondary immune modulators participating in down-regulation of inflammatory processes (17, 43). The best characterized members of endocannabinoid lipid mediators are N-arachidonoylethanolamine (anandamide, AEA) and 2-arachidonoylglycerol (2-AG) (44, 45), which are derived from membrane phospholipids in response to physiological or pathological stimuli. Furthermore, new signaling mechanisms for intracellular transport and storage of endocannabinoids have been described (46–49). Endocannabinoids act through type-1 (CB1) and type-2 (CB2) G protein-coupled cannabinoid receptors, G protein-coupled receptor GPR55, transient receptor potential channel of the vanilloid subfamily 1 (TRPV1), and peroxisome proliferator-activated receptor γ (PPAR γ) (50). CB1 and CB2 are expressed on MCs (51) and initiate a series of signal transduction events that converge at the transcriptional level to regulate cell migration and production of cytokines and chemokines (52, 53). Acting in concert with GPR55, CB2 mediates signals inhibiting MC degranulation and cytokine synthesis (54). Described anti-fibrotic effects of cannabinoid receptors in different MC-related disease models (55, 56) together with the recently deciphered crystal structure of CB1 and CB2 (57, 58) will allow development of selective agonists and their implementation in novel therapeutic concepts for allergic diseases.

ALLERGY-ASSOCIATED IMMUNOMODULATORY LIPIDS ACT ON MAST CELLS

One would expect that allergen-associated lipids of plants or bacterial origin preferentially affect epithelial cells. Interaction of lipids with MCs might rather be possible in tissues with a damaged barrier (mainly by proteolytic activity of allergens) or indirectly in individuals showing previous sensitization

TABLE 1 | Lipid categories and examples of lipids affecting MC reactivity.

Lipid category	Schematic structure	Examples of lipids with effects on MC reactivity	References
Fatty acyls	 α-Linolenic acid	Fatty acids, Omega 3, and 6 polyunsaturated fatty acids	(10–13)
		PG, TX, LT, LX	(14–16)
		AEA	(17)
Glycerolipids	 Triglyceride	2-AG	(17)
Glycerophospholipids	 Phosphatidylethanolamine	PC, PE, PI, PS, PAF	(18–20)
Sphingolipids	 Sphingomyelin	Ceramide	(21)
		C1P, S1P	(22, 23)
Sterol lipids	 Cholesterol	Cholesterol	(24–27)
		Steroids	(28)
		Vitamin D_3	(29, 30)
Prenol lipids	 Vitamin A	Carotenoids	(11)
		Vitamin E	(31)
Saccharolipds	 Lipid A -disaccharide-1-phosphate	LPS	(32, 33)
Polyketides	 Griseorhodin A	Antibiotics	(34)
		Flavonoides	(11)

http://www.lipidmaps.org is a source for structure of lipids. TX, thromboxane; PI, phosphatidylinositol.

and presence of specific IgE and active transport of IgE-antigen complexes containing lipids. MCs are regulated by lipids associated with different allergens, as extensively reviewed elsewhere (59, 60). Interestingly, epithelial cells from healthy donors sense allergens differently than epithelial cells from allergic patients (61). Therefore, enhanced reactivity of MCs could be the result of a combined action of allergen-lipid complexes and pro-allergic inflammatory mediators produced by epithelial cells (**Figure 1**). Degranulation of human lung MCs has been shown to be inhibited in coculture with bronchial epithelial cells (62), substantiating the hypothesis that epithelial cells can provide the inhibitory signals to MCs as well. Attractive candidates potentially limiting MC-reactivity are specialized pro-resolving mediators (SPMs) crucial for the resolution of inflammatory processes (63, 64). Four classes of SPMs have been characterized so far. Lipoxins (LX) are biosynthetic products of arachidonic acid. Resolvins, protectins, and maresins are products of eicosapentaenoic acid (EPA), docosapentaenoic acid (n-3DPA) or docosahexaenoic acid (DHA) (64). The epithelial cell-derived resolvins D1, D2, and lipoxin A4 have been found to suppress IgE-mediated histamine release from MCs via G-protein-coupled receptors (65). Furthermore, airway inflammation, mucus production, and airway hyperresponsiveness *in vivo* as well as MC degranulation and cytokine release were decreased by lipoxin B4 application (16), indicating therapeutic potential of pro-resolving lipid mediators in regulation of MC reactivity. Whether SPMs could be produced by MCs themselves, is unknown.

One major class of allergen-containing particles represent plant pollens, where pollen grains are coated with different lipids

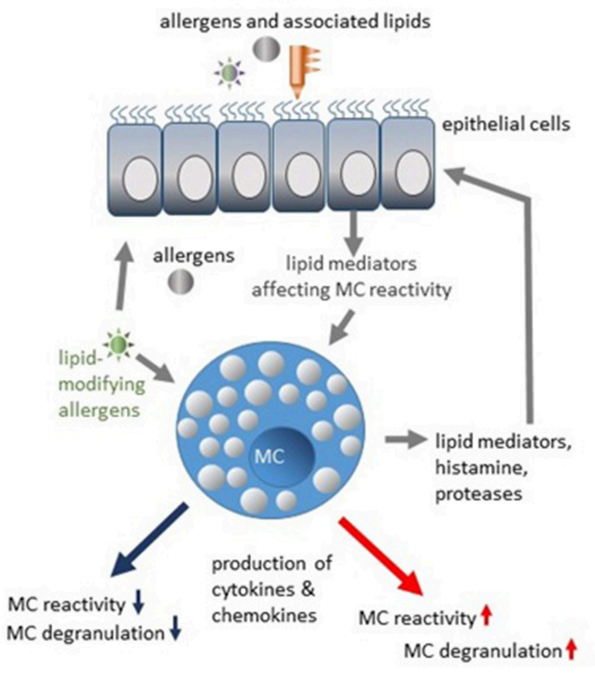


FIGURE 1 | Modulation of MC reactivity by allergen-associated lipids and lipid mediators.

essential for plant fertilization (66). By interacting with immune cells and epithelial cells, pollen lipids may play an important role in immunoregulation. Two classes of pollen-associated lipid mediators (PALMs) have been described so far, namely LTB4-like mediators, which are monoxydroxylated derivatives of linoleic acid, and phytoprostanes generated from α-linolenic acid in response to oxidative stress (67). Effects of PALMs on MC degranulation have been reported for aqueous pollen extracts (APE) derived from birch pollen and for APE from *Ambrosia artemisiifolia* (68). Here, degranulation was induced in skin MCs of C57BL/6 mice by intradermal injection of APE in the absence of specific IgE. It is not known whether MC degranulation can be induced by APE themselves in the presence of an intact skin barrier. In experiments with RBL cells, it has been shown that *Ambrosia* pollen extract induces histamine release by a ROS-dependent mechanisms, but not β-hexosaminidase release (69). In experiments with mountain ceder (*Juniperus ashei*) pollen extract, release of both serotonin and β-hexosaminidase was induced in RBL cells in an IgE-independent, but ROS-dependent manner. Added to suboptimal IgE/AG concentrations, pollen extracts enhanced degranulation of RBL cells (70), although lipid components in particular extracts were not analyzed. Interestingly, persistent contact with grass pollen in early childhood has been found to represent one of various allergy-protective factors (71). However, whether lipids are essential for tolerance induction and whether MCs are directly involved in tolerance development remains to be investigated.

ALLERGENS INDUCE CHANGES IN MAST CELL LIPID COMPOSITION

Interaction of honeybee venom phospholipase A2 (PLA2) with membrane lipids is an example how allergen-induced modification of lipids could tune MC reactivity. Insect venom, particularly *Hymenoptera* venoms, induces a pronounced Th2 response by coopting evolutionary conserved immunological and neurological mechanisms (3, 72). A mixture of different substances, including enzymes, toxic peptides, lipids, and biogenic amines, is transported into the skin by the insect sting and induces a local inflammatory reaction, leading to sensitization and IgE production. Phospholipase A2 is one of the two major honeybee (*Apis mellifera*) venom allergens (73, 74). Cleaving cell membrane phosphodiacylglycerides, PLA2 induces the release of lysophospholipids, particularly lysopho sphatidylcholine (LPC), together with fatty acids. This local lipid remodeling can affect MC reactivity *per se* and lead to MC degranulation (75, 76) (**Figure 1**). The stimulatory effect on MCs is absent if the enzymatically inactive form of PLA2 is used (77). PLA2 enzymatic activity is also required to induce a Th2 response (78). Generated neoantigens in the skin are presented by the CD1a molecules of antigen-presenting cells (APCs) and then induce a polyclonal T cell response (79). Interestingly, the stimulatory effects of PLA2 were observed only in the presence of lipids, either venom- or host-derived, indicating that lipid and protein components act in concert to induce a T cell response (79). PLA2 activity has also been detected

in house dust mite extracts (80), indicating that generation of lysophospholipids could be a part of allergic sensitization program. Moreover, an interaction between MCs and dendritic cells (DCs) has been demonstrated in contact hypersensitivity models (81, 82), where MCs were "cross-dressed" with DC MHC class II complexes. How the local changes of lipid composition, induced by e.g., PLA2 activity, modulate the MC-DC interaction and which functional consequences this would have for the T cell response in allergic settings remains to be further elucidated.

MEMBRANE LIPID ORGANIZING ENZYMES ARE TARGETS TO MODULATE MAST CELL ACTIVATION

Organization of membrane lipids plays an important role in regulation of MC degranulation. The inner leaflet, facing the inside of the cell, contains negatively charged amino-phospholipids, and phosphatidylethanolamine (PE). The outer leaflet, facing the outside environment, contains phosphatidylcholine (PC) and sphingomyelin. Asymmetric distribution of phospholipids in the plasma membrane plays an essential role in regulation of MC exocytosis (83). Interestingly, one of the earliest events in MC degranulation is a redistribution of phosphatidylinositol 4,5-bisphospate [PtdIns(4,5)*P2*] disappearing from the plasma membrane within seconds after stimulation (84). Furthermore, MC degranulation is associated with reversible phosphatidylserine (PS) translocation to the plasma membrane (85), in contrast to various other cell types, in which the PS translocation represents an apoptotic "eat-me" signal. PS exposure can implicate endocytosis, acquisition of membrane curvature, regulation of transmembrane proteins, interactions with cytoskeletal elements as well as involvement in PS signaling (19). Lipid transporting phospholipid scramblase 1 (PLSCR1), floppase ABCA1 or transmembrane protein TMEM16F are the candidates responsible for PS translocation (19, 86). Interestingly, PS translocation in MCs could be induced not only by FcεRI-mediated activation. Crosslinking of glycosylphosphatidylinositol-anchored proteins by specific antibodies or lectins also induce PS externalization, using probably a different Ca^{2+}-independent mechanism (87). It seems that the context in which MCs recognize PS is important, since free PS and lyso-PS enhance FcεRI-mediated degranulation (88) and phosphatidylserine-specific phospholipase A1, released e.g., by activated platelets, generates lyso-PS and strongly enhances MC histamine release (75). However, recognition of PS on the surface of apoptotic cells by the inhibitory receptor CD300a leads to a downregulation of inflammatory cytokine and chemokine production (89). Also, rodent MCs express α-galactosyl derivatives of the ganglioside GD1b (90). Antibodies recognizing this ganglioside inhibit degranulation and histamine release by modulating FcεRI endocytosis (90, 91) but in contrast, are also able to promote release of cytokines and lipid mediators (92).

Lipid content and distribution in membranes are regulated by different enzymes. Three types of phospholipid transportation enzymes are responsible for maintenance of the phospholipid asymmetry in membranes: (1) flippases that catalyze translocations of phospholipids between membrane leaflets in an energy-dependent or-independent manner, primarily from the external to the internal leaflet, (2) floppases that transport lipids from the cytoplasmic leaflet to the external membrane leaflet, and (3) scramblases that move lipids between the two leaflets [as reviewed in Pomorski and Menon (93)]. Potential effects of flippases, floppases, and scramblases on MC function are outlined in **Figure 2**.

Flippases are members of the P4-type ATPase family with a similar structure containing 10 transmembrane domains, an actuator domain, a phosphorylation domain, and a nucleotide-binding domain associating with an accessory subunit Cdc50, forming a heterodimeric complex. In mammals, 14 different P4-ATPases have been identified as heterodimers consisting of a catalytic subunit in association with one member of the Cdc50 family (94). Many P4-ATPases are ubiquitously expressed and have been implicated in different metabolic diseases (95). P4-ATPases are also involved in the phospholipid transport between different subcellular compartments and are responsible for maintenance of phospholipid asymmetry in different cell types. Lipid transport by P4-ATPases is lipid-specific, head group-, and backbone-dependent (96). P4-ATPases regulate vesicular trafficking and the bidirectional vesicular transport between

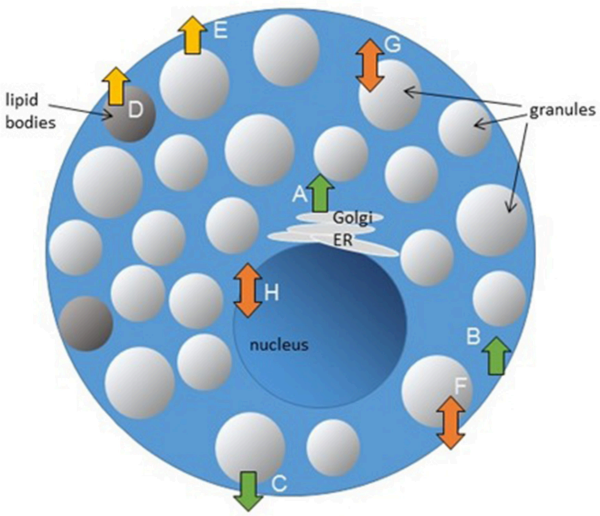


FIGURE 2 | Potential effects of flippases, floppases, and scramblases on MC function. Lipid transporting enzymes could be involved in regulation of different cellular processes. Granule biogenesis **(A)**, endocytosis **(B)**, and exocytosis **(C)** could be regulated by flippases. Floppases are participating in transport of lipid mediators **(D)** and could be involved in regulation of MC exocytosis **(E)**. Scramblases regulate FcεRI-mediated signaling and MC degranulation **(F)**, could be potentially involved in TLR-signaling in endosomes **(G)** and regulation of gene transcription **(H)**.

the Golgi complex and early endosomes, but also vesicle biogenesis by enriching specific phospholipids in yeast cells, formation of post-Golgi vesicles in plant cells, as well as maintenance of membrane integrity and secretory processes (95, 97). Involvement of this class of lipid transporters in MC granule biogenesis and exocytosis is obvious. Genetic models with MC-specific P4-ATPase inactivation/overexpression could help to better understand essential regulatory steps in granule development, maturation and degranulation of MCs. Improvement of the knowledge on MC granule organization will also help to develop new strategies to interfere with MC degranulation.

Lipid transporters, shipping lipids from the inner membrane leaflet to the outer leaflet, are ATP-biding cassette (ABC) proteins, originally identified as multidrug resistance gene products in tumor cells. ABC proteins are encoded as single polypeptides, which can form homo- or heterodimers, contain an ATP-binding site, a nucleotide-binding domain and up to 17 transmembrane domains (98). Leukotriene C4 was the first lipid mediator described, transported by ABCC1/MRP1 (99). Different other lipid mediators such as prostaglandin A2 (PGA2) and 15-deoxy-$\Delta^{(12,14)}$ PGJ2, lysophosphatidylinositol (LPI) are also transported by specific ABC proteins (100, 101). In MCs with downregulated ABCC1/MRP1 expression, transport of S1P was strongly reduced, indicating an ABC-dependent regulation of MC chemotaxis and migration (102). How the lipid transport can be modulated by targeting other ABC proteins and how this will affect MC function is currently unknown. Mutations of ABC lipid transporters are responsible for several human diseases, such as neonatal surfactant deficiency (ABCA3 mutation) or Tangier disease (ABCA1 mutation), characterized by decreased removal of cholesterol from peripheral tissues (103).

Scramblases are structurally related proteins, containing a DNA-binding domain, a palmitoylation motif, a Ca^{2+}-binding motif, transmembrane domains and a nuclear localization signal. Located in the plasma membrane, scramblases are involved in the Ca^{2+}-dependent distribution of phospholipids (scrambling) (104). In 2008, the group of Benhamou identified phospholipid scramblase 1 (PLSCR1) as specific regulator of FcεRI signaling (105). Initially, PLSCR1 was only marked responsible for the rapid redistribution of phospholipids between two leaflets of the plasma membrane after cell activation or apoptosis, leading to the disruption of their asymmetric distribution (106–108). In the meantime, however, it is known that PLSCR1 serves numerous functions beyond the redistribution of phospholipids, such as the regulation of cell proliferation, differentiation, apoptosis, and tumor development (109–112). PLSCR1 requires palmitoylation to be stabilized at the plasma membrane. In the absence of palmitoylation, it is found in the nucleus, where it can bind DNA and activate the transcription of the inositol phosphate-3 (IP3) receptor (113, 114). When palmitoylated and localized at the plasma membrane, it participates in the epidermal growth factor signaling (115) by amplifying activation of the tyrosine kinase Src (116).

Knock-down of PLSCR1 in RBL-2H3 cells significantly impaired FcεRI-mediated degranulation and release of vascular endothelial growth factor (105). Earlier, Pastorelli had already observed that phosphorylation of PLSCR1 is increased following the engagement of FcεRI in RBL-2H3 cells (117). Tyrosine phosphorylation of PLSCR1 following FcεRI aggregation relies on Lyn and Syk tyrosine kinases and partially also on calcium mobilization. In contrast, Fyn signaling negatively regulated PLSCR1 phosphorylation, suggesting a complex modulation of FcεRI-dependent MC activation by PLSCR1 (105, 118). *In vivo* studies using *Plscr1*$^{-/-}$ mice showed reduced FcεRI-dependent passive systemic anaphylaxis and serum histamine levels compared to wild-type mice (119), demonstrating the involvement of PLSCR1 in IgE-mediated anaphylaxis without affecting the phenotype or tissue distribution of resting MCs. Surprisingly, anaphylactic reactions induced by direct injection of histamine were slightly increased in *Plscr1*$^{-/-}$ animals, indicating that PLSCR1 also counter-regulates IgE-dependent anaphylaxis at later stages.

The modulatory ability of PLSCR1, allowing increased as well as decreased biological responses, might serve to sophisticatedly regulate inflammation, host defense, tissue remodeling and homoeostasis and provide a rationale for exploiting PLSCR1 as therapeutic target in allergies (119, 120). Interestingly, in plasmacytoid DCs, PLSCR1 interacts with TLR9, and regulates the type I IFN response by modulating endosomal trafficking of TLR9 (121). Also, scramblase 2 (PLSCR2) has been found to be involved in the antiviral response. PLSCR2 binds to STAT3 and in this way also participates in downregulating the type I interferon response (122). Whether comparable effects will be observed in MCs and whether the antiviral response of MCs is compromised in the absence of scramblases remains an open question for future investigations. Mice deficient for scramblase 3 developed metabolic syndrome and lipid accumulation in abdominal fat pads (123).

Modulation of MC degranulation by affecting the lipid composition of the cell membrane or the enzyme activity modulating the lipid distribution of the membrane are potential emerging therapeutic strategies for the treatment of allergic diseases.

CONCLUSION AND PERSPECTIVES

The rapidly emerging field defining modulation of MC reactivity by lipids, in addition to proteins, reveals novel and unprecedented targets, which may serve to preclude MC effects in allergic reactions. Active substances secreted by MCs have already been studied extensively, but data on the overall lipid composition of MCs and on stimulus-specific as well as sub-cell type-specific lipidomic data are still missing. Direct effects of immunomodulatory lipids on MC degranulation, changes in MC lipid composition induced by allergens themselves and changes in lipid transport and metabolism in MCs have not yet been comprehensively investigated. Furthermore, current studies investigating MC lipids are often limited by use of non-physiologic conditions or narrow restriction of the lipids that were analyzed. Therefore, addition of modern lipidomic approaches to the toolbox of immunology and cell

biology is crucial. Hereby, the added knowledge of lipid production and regulation together with deep understanding of MC biology will help find new mechanisms regulating MC responses. Coupled with this, an in-depth knowledge will be considerably advantageous for patients with anaphylaxis, asthma, allergic rhinitis, eczema, urticaria, mastocytosis, and other allergic diseases.

AUTHOR CONTRIBUTIONS

PH, SN-D, JH, KH, and ZO wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

FUNDING

This work was supported by the Research Center Borstel to PH and ZO, the Medical Faculty of the University of Luebeck to JH and KH, and the German Research Foundation (DFG), Project HA 2393/6-1 to KH, Research Training Group 1727 "Modulation of Autoimmunity" (RTG 1727) to PH, SN-D, JH, KH, ZO, and Excellence Cluster 306 "Inflammation at Interfaces" (EXC 306) to PH and KH.

ACKNOWLEDGMENTS

We thank Prof. Dr. Heinz Fehrenbach for continuous support.

REFERENCES

1. Mukai K, Tsai M, Saito H, Galli SJ. Mast cells as sources of cytokines, chemokines, and growth factors. *Immunol Rev*. (2018) 282:121–50. doi: 10.1111/imr.12634
2. Wernersson S, Pejler G. Mast cell secretory granules: armed for battle. *Nat Rev Immunol*. (2014) 14:478–94. doi: 10.1038/nri3690
3. Palm NW, Rosenstein RK, Medzhitov R. Allergic host defences. *Nature*. (2012) 484:465–72. doi: 10.1038/nature11047
4. Storck EM, Özbalci C, Eggert US. Lipid cell biology: a focus on lipids in cell division. *Ann Rev Biochem*. (2018) 87:839–69. doi: 10.1146/annurev-biochem-062917-012448
5. Litvinov DY, Savushkin EV, Dergunov AD. Intracellular and plasma membrane events in cholesterol transport and homeostasis. *J Lipids*. (2018) 2018:1–22. doi: 10.1155/2018/3965054
6. van Meer G, de Kroon AIPM. Lipid map of the mammalian cell. *J Cell Sci*. (2011) 124:5–8. doi: 10.1242/jcs.071233
7. van Meer G, Voelker DR, Feigenson GW. Membrane lipids: where they are and how they behave. *Nat Rev Mol Cell Biol*. (2008) 9:112–24. doi: 10.1038/nrm2330
8. Thelen AM, Zoncu R. Emerging roles for the lysosome in lipid metabolism. *Trends Cell Biol*. (2017) 27:833–50. doi: 10.1016/j.tcb.2017.07.006
9. Márquez MG, Favale NO, Leocata Nieto F, Pescio LG, Sterin-Speziale N. Changes in membrane lipid composition cause alterations in epithelial cell–cell adhesion structures in renal papillary collecting duct cells. *Biochim Biophy Acta*. (2012) 1818:491–501. doi: 10.1016/j.bbamem.2011.11.018
10. Gueck T, Seidel A, Fuhrmann H. Effects of essential fatty acids on mediators of mast cells in culture. *Prostagl Leukotr Essent Fatty Acids*. (2003) 68:317–22. doi: 10.1016/S0952-3278(03)00022-X
11. Hagenlocher Y, Lorentz A. Immunomodulation of mast cells by nutrients. *Mol Immunol*. (2015) 63:25–31. doi: 10.1016/j.molimm.2013.12.005
12. Ju H-R, Wu H-Y, Nishizono S, Sakono M, Ikeda I, Sugano M, et al. Effects of dietary fats and curcumin on IgE-mediated degranulation of intestinal mast cells in brown norway rats. *Biosci Biotechnol Biochem*. (1996) 60:1856–60. doi: 10.1271/bbb.60.1856
13. Wang X, Kulka M. n-3 Polyunsaturated fatty acids and mast cell activation. *J Leukocyte Biol*. (2015) 97:859–71. doi: 10.1189/jlb.2RU0814-388R
14. Samuelsson B, Dahlen S, Lindgren J, Rouzer C, Serhan C. Leukotrienes and lipoxins: structures, biosynthesis, and biological effects. *Science*. (1987) 237:1171–6. doi: 10.1126/science.2820055
15. Boyce JA. Mast cells and eicosanoid mediators: a system of reciprocal paracrine and autocrine regulation. *Immunol Rev*. (2007) 217:168–85. doi: 10.1111/j.1600-065X.2007.00512.x
16. Karra L, Haworth O, Priluck R, Levy BD, Levi-Schaffer F. Lipoxin B4 promotes the resolution of allergic inflammation in the upper and lower airways of mice. *Mucosal Immunol*. (2015) 8:852–62. doi: 10.1038/mi.2014.116
17. Chiurchiù V, Battistini L, Maccarrone M. Endocannabinoid signalling in innate and adaptive immunity. *Immunology*. (2015) 144:352–64. doi: 10.1111/imm.12441
18. Byrne R, Larijani B. The role of phosphoinositides in mast cell signalling. *Signal Trans*. (2006) 6:81–91. doi: 10.1002/sita.200500074
19. Shimoda LMN, Dixon AM, Speck M, Stokes AJ, Turner H, Umemoto EY. Beyond apoptosis: the mechanism and function of phosphatidylserine asymmetry in the membrane of activating mast cells. *BioArchitecture*. (2014) 4:127–37. doi: 10.1080/19490992.2014.995516
20. Schauberger E, Peinhaupt M, Cazares T, Lindsley AW. Lipid mediators of allergic disease: pathways, treatments, and emerging therapeutic targets. *Curr Allergy Asthma Rep*. (2016) 16:48. doi: 10.1007/s11882-016-0628-3
21. Chiba N, Masuda A, Yoshikai Y, Matsuguchi T. Ceramide inhibits LPS-induced production of IL-5, IL-10, and IL-13 from mast cells. *J Cell Physiol*. (2007) 213:126–36. doi: 10.1002/jcp.21101
22. Olivera A, Rivera J. Sphingolipids and the balancing of immune cell function: lessons from the mast cell. *J Immunol*. (2005) 174:1153–8. doi: 10.4049/jimmunol.174.3.1153
23. Sturgill JL. Sphingolipids and their enigmatic role in asthma. *Adv Biol Regul*. (2018) 70:74–81. doi: 10.1016/j.jbior.2018.09.001
24. Baumruker T. Activation of mast cells by incorporation of cholesterol into rafts. *Int Immunol*. (2003) 15:1207–18. doi: 10.1093/intimm/dxg120
25. Kato N, Nakanishi M, Hirashima N. Cholesterol depletion inhibits store-operated calcium currents and exocytotic membrane fusion in RBL-2H3 cells. *Biochemistry*. (2003) 42:11808–14. doi: 10.1021/bi034758h
26. Surviladze Z, Dráberová L, Kovárová M, Boubelík M, Dráber P. Differential sensitivity to acute cholesterol lowering of activation mediated via the high-affinity IgE receptor and Thy-1 glycoprotein. *Eur J Immunol*. (2001) 31:1–10. doi: 10.1002/1521-4141(200101)31:1<1::AID-IMMU1>3.0.CO;2-W
27. Kovarova M, Wassif CA, Odom S, Liao K, Porter FD, Rivera J. Cholesterol deficiency in a mouse model of Smith-Lemli-Opitz syndrome reveals increased mast cell responsiveness. *J Exp Med*. (2006) 203:1161–71. doi: 10.1084/jem.20051701
28. Mori T, Abe N, Saito K, Toyama H, Endo Y, Ejima Y, et al. Hydrocortisone and dexamethasone dose-dependently stabilize mast cells derived from rat peritoneum. *Pharmacol Rep*. (2016) 68:1358–65. doi: 10.1016/j.pharep.2016.09.005
29. Liu Z-Q, Li X-X, Qiu S-Q, Yu Y, Li M-G, Yang L-T, et al. Vitamin D contributes to mast cell stabilization. *Allergy*. (2017) 72:1184–92. doi: 10.1111/all.13110
30. Yip K-H, Kolesnikoff N, Yu C, Hauschild N, Taing H, Biggs L, et al. Mechanisms of vitamin D3 metabolite repression of IgE-dependent mast cell activation. *J Allergy Clini Immunol*. (2014) 133:1356–64.e14. doi: 10.1016/j.jaci.2013.11.030
31. Zingg J. Vitamin E and mast cells. In: Lidwack G, editor. *Vitamins and Hormones*. Amsterdam: Elsevier. p. 393–418. doi: 10.1016/S0083-6729(07)76015-6
32. Masuda A, Yoshikai Y, Aiba K, Matsuguchi T. Th2 cytokine production from mast cells is directly induced by lipopolysaccharide and distinctly

regulated by c-Jun N-terminal kinase and p38 pathways. *J Immunol*. (2002) 169:3801–0. doi: 10.4049/jimmunol.169.7.3801
33. Varadaradjalou S, Féger F, Thieblemont N, Hamouda NB, Pleau J-M, Dy M, et al. Toll-like receptor 2 (TLR2) and TLR4 differentially activate human mast cells. *Eur J Immunol*. (2003) 33:899–906. doi: 10.1002/eji.2003 23830
34. Kazama I, Saito K, Baba A, Mori T, Abe N, Endo Y, et al. Clarithromycin dose-dependently stabilizes rat peritoneal mast cells. *Chemotherapy*. (2016) 61:295–303. doi: 10.1159/000445023
35. Kulinski JM, Muñoz-Cano R, Olivera A. Sphingosine-1-phosphate and other lipid mediators generated by mast cells as critical players in allergy and mast cell function. *Eur J Pharmacol*. (2016) 778:56–67. doi: 10.1016/j.ejphar.2015.02.058
36. Bankova LG, Lai J, Yoshimoto E, Boyce JA, Austen KF, Kanaoka Y, et al. Leukotriene E_4 elicits respiratory epithelial cell mucin release through the G-protein–coupled receptor, GPR99. *Proc Natl Acad Sci USA*. (2016) 113:6242–7. doi: 10.1073/pnas.1605957113
37. Samuchiwal SK, Boyce JA. Role of lipid mediators and control of lymphocyte responses in type 2 immunopathology. *J Allergy Clini Immunol*. (2018) 141:1182–90. doi: 10.1016/j.jaci.2018.02.006
38. Nakamura T, Fujiwara Y, Yamada R, Fujii W, Hamabata T, Lee MY, et al. Mast cell–derived prostaglandin D 2 attenuates anaphylactic reactions in mice. *J Allergy Clini Immunol*. (2017) 140:630–2.e9. doi: 10.1016/j.jaci.2017.02.030
39. Shimanaka Y, Kono N, Taketomi Y, Arita M, Okayama Y, Tanaka Y, et al. Omega-3 fatty acid epoxides are autocrine mediators that control the magnitude of IgE-mediated mast cell activation. *Nat Med*. (2017) 23:1287–97. doi: 10.1038/nm.4417
40. The Immunological Genome Project Consortium, Dwyer DF, Barrett NA, Austen KF. Expression profiling of constitutive mast cells reveals a unique identity within the immune system. *Nat Immunol*. (2016) 17:878–87. doi: 10.1038/ni.3445
41. Lundström SL, Saluja R, Adner M, Haeggström JZ, Nilsson G, Wheelock CE. Lipid mediator metabolic profiling demonstrates differences in eicosanoid patterns in two phenotypically distinct mast cell populations. *J Lipid Res*. (2013) 54:116–26. doi: 10.1194/jlr.M030171
42. Olivera A, Rivera J. An emerging role for the lipid mediator sphingosine-1-phosphate in mast cell effector function and allergic disease. *Mast Cell Biol*. (2011) 716:123–42. doi: 10.1007/978-1-4419-9533-9_8
43. Migalovich-Sheikhet H, Friedman S, Mankuta D, Levi-Schaffer F. Novel identified receptors on mast cells. *Front Immunol*. (2012) 3:238. doi: 10.3389/fimmu.2012.00238
44. Devane W, Hanus L, Breuer A, Pertwee R, Stevenson L, Griffin G, et al. Isolation and structure of a brain constituent that binds to the cannabinoid receptor. *Science*. (1992) 258:1946–9. doi: 10.1126/science.1470919
45. Mechoulam R, Ben-Shabat S, Hanus L, Ligumsky M, Kaminski NE, Schatz AR, et al. Identification of an endogenous 2-monoglyceride, present in canine gut, that binds to cannabinoid receptors. *Biochem Pharmacol*. (1995) 50:83–90. doi: 10.1016/0006-2952(95)00109-D
46. Oddi S, Fezza F, Pasquariello N, De Simone C, Rapino C, Dainese E, et al. Evidence for the intracellular accumulation of anandamide in adiposomes. *Cell Mol Life Sci*. (2008) 65:840–50. doi: 10.1007/s00018-008-7494-7
47. Oddi S, Fezza F, Pasquariello N, D'Agostino A, Catanzaro G, De Simone C, et al. Molecular identification of albumin and Hsp70 as cytosolic anandamide-binding proteins. *Chem Biol*. (2009) 16:624–32. doi: 10.1016/j.chembiol.2009.05.004
48. Kaczocha M, Glaser ST, Chae J, Brown DA, Deutsch DG. Lipid droplets are novel sites of *N*-acylethanolamine Inactivation by fatty acid amide hydrolase-2. *J Biol Chem*. (2010) 285:2796–806. doi: 10.1074/jbc.M109.058461
49. Maccarrone M, Dainese E, Oddi S. Intracellular trafficking of anandamide: new concepts for signaling. *Trends Biochem Sci*. (2010) 35:601–8. doi: 10.1016/j.tibs.2010.05.008
50. Pertwee R. Receptors and channels targeted by synthetic cannabinoid receptor agonists and antagonists. *Curr Med Chem*. (2010) 17:1360–81. doi: 10.2174/092986710790980050
51. Samson M-T, Small-Howard A, Shimoda LMN, Koblan-Huberson M, Stokes AJ, Turner H. Differential roles of CB1 and CB2 cannabinoid receptors in mast cells. *J Immunol*. (2003) 170:4953–62. doi: 10.4049/jimmunol.170.10.4953
52. Sugawara K, Zákány N, Hundt T, Emelianov V, Tsuruta D, Schäfer C, et al. Cannabinoid receptor 1 controls human mucosal-type mast cell degranulation and maturation *in situ*. *J Allergy Clini Immunol*. (2013) 132:182–93.e8. doi: 10.1016/j.jaci.2013.01.002
53. Sugawara K, Bíró T, Tsuruta D, Tóth BI, Kromminga A, Zákány N, et al. Endocannabinoids limit excessive mast cell maturation and activation in human skin. *J Allergy Clini Immunol*. (2012) 129:726–38.e8. doi: 10.1016/j.jaci.2011.11.009
54. Cruz SL, Sánchez-Miranda E, Castillo-Arellano JI, Cervantes-Villagrana RD, Ibarra-Sánchez A, González-Espinosa C. Anandamide inhibits FcεRI-dependent degranulation and cytokine synthesis in mast cells through CB2 and GPR55 receptor activation. Possible involvement of CB2-GPR55 heteromers. *Int Immunopharm*. (2018) 64:298–307. doi: 10.1016/j.intimp.2018.09.006
55. Zhou L, Zhou S, Yang P, Tian Y, Feng Z, Xie X-Q, et al. Targeted inhibition of the type 2 cannabinoid receptor is a novel approach to reduce renal fibrosis. *Kidney Int*. (2018) 94:756–72. doi: 10.1016/j.kint.201 8.05.023
56. Vuolo F, Abreu SC, Michels M, Xisto DG, Blanco NG, Hallak JE, et al. Cannabidiol reduces airway inflammation and fibrosis in experimental allergic asthma. *Eur J Pharmacol*. (2019) 843:251–9. doi: 10.1016/j.ejphar.2018.11.029
57. Krishna Kumar K, Shalev-Benami M, Robertson MJ, Hu H, Banister SD, Hollingsworth SA, et al. Structure of a signaling cannabinoid receptor 1-G protein complex. *Cell*. (2019) 176:448–58.e12. doi: 10.1016/j.cell.2018.11.040
58. Li X, Hua T, Vemuri K, Ho J-H, Wu Y, Wu L, et al. Crystal structure of the human cannabinoid receptor CB2. *Cell*. (2019) 176:459–67.e13. doi: 10.1016/j.cell.2018.12.011
59. Bublin M, Eiwegger T, Breiteneder H. Do lipids influence the allergic sensitization process? *J Allergy Clini Immunol*. (2014) 134:521–9. doi: 10.1016/j.jaci.2014.04.015
60. del Moral MG, Martínez-Naves E. The role of lipids in development of allergic responses. *Imm Netw*. (2017) 17:133. doi: 10.4110/in.2017.17.3.133
61. Mattila P, Renkonen J, Toppila-Salmi S, Parviainen V, Joenväärä S, Alff-Tuomala S, et al. Time-series nasal epithelial transcriptomics during natural pollen exposure in healthy subjects and allergic patients. *Allergy*. (2010) 65:175–83. doi: 10.1111/j.1398-9995.2009.02181.x
62. Yang W, Wardlaw AJ, Bradding P. Attenuation of human lung mast cell degranulation by bronchial epithelium. *Allergy*. (2006) 61:569–75. doi: 10.1111/j.1398-9995.2006.01041.x
63. Basil MC, Levy BD. Specialized pro-resolving mediators: endogenous regulators of infection and inflammation. *Nat Rev Immunol*. (2015) 16:51. doi: 10.1038/nri.2015.4
64. Serhan CN, Levy BD. Resolvins in inflammation: emergence of the pro-resolving superfamily of mediators. *J Clin Invest*. (2018) 128:2657–69. doi: 10.1172/JCI97943
65. Martin N, Ruddick A, Arthur GK, Wan H, Woodman L, Brightling CE, et al. Primary human airway epithelial cell-dependent inhibition of human lung mast cell degranulation. *PLoS ONE*. (2012) 7:e43545. doi: 10.1371/journal.pone.0043545
66. Bashir MEH, Lui JH, Palnivelu R, Naclerio RM, Preuss D. Pollen lipidomics: lipid profiling exposes a notable diversity in 22 allergenic pollen and potential biomarkers of the allergic immune response. *PLoS ONE*. (2013) 8:e57566. doi: 10.1371/journal.pone.0057566
67. Gilles S, Behrendt H, Ring J, Traidl-Hoffmann C. The pollen enigma: modulation of the allergic immune response by non-allergenic, pollen-derived compounds. *Curr Pharm Design*. (2012) 18:2314–9. doi: 10.2174/138161212800166040
68. Metz M, Gilles S, Geldmacher A, Behrendt H, Traidl-Hoffmann C, Maurer M. Evidence for non-allergic mast cell activation in pollen-associated inflammation. *J Invest Dermatol*. (2011) 131:987–90. doi: 10.1038/jid.2010.419
69. Chodaczek G, Bacsi A, Dharajiya N, Sur S, Hazra TK, Boldogh I. Ragweed pollen-mediated IgE-independent release of biogenic amines from mast cells via induction of mitochondrial dysfunction. *Mol Immunol*. (2009) 46:2505–14. doi: 10.1016/j.molimm.2009.05.023
70. Endo S, Hochman DJ, Midoro-Horiuti T, Goldblum RM, Brooks EG. Mountain cedar pollen induces IgE-independent mast cell degranulation,

IL-4 production, and intracellular reactive oxygen species generation. *Cell Immunol*. (2011) 271:488–95. doi: 10.1016/j.cellimm.2011.08.019
71. Sudre B, Vacheyrou M, Braun-Fahrländer C, Normand A-C, Waser M, Reboux G, et al. High levels of grass pollen inside European dairy farms: a role for the allergy-protective effects of environment? *Allergy*. (2009) 64:1068–73. doi: 10.1111/j.1398-9995.2009.01958.x
72. Elieh Ali Komi D, Shafaghat F, Zwiener RD. Immunology of bee venom. *Clini Rev Allergy Immunol*. (2018) 54:386–96. doi: 10.1007/s12016-017-8597-4
73. Bilò MB, Antonicelli L, Bonifazi F. Honeybee venom immunotherapy: certainties and pitfalls. *Immunotherapy*. (2012) 4:1153–66. doi: 10.2217/imt.12.113
74. Müller UR. Insect venoms. In: Ring J, editor. *Chemical Immunology and Allergy* (Basel: KARGER). p. 141–156. doi: 10.1159/000315948
75. Hosono H, Aoki J, Nagai Y, Bandoh K, Ishida M, Taguchi R, et al. Phosphatidylserine-specific phospholipase A1 stimulates histamine release from rat peritoneal mast cells through production of 2-acyl-1-lysophosphatidylserine. *J Biol Chem*. (2001) 276:29664–70. doi: 10.1074/jbc.M104597200
76. Murakami M, Hara N, Kudo I, Inoue K. Triggering of degranulation in mast cells by exogenous type II phospholipase A2. *J Immunol*. (1993) 151:5675.
77. Dudler T, Machado DC, Kolbe L, Annand RR, Rhodes N, Gelb MH, et al. A link between catalytic activity, IgE-independent mast cell activation, and allergenicity of bee venom phospholipase A2. *J Immunol*. (1995) 155:2605.
78. Palm NW, Rosenstein RK, Yu S, Schenten DD, Florsheim E, Medzhitov R. Bee venom phospholipase A2 induces a primary type 2 response that Is dependent on the receptor ST2 and confers protective immunity. *Immunity*. (2013) 39:976–85. doi: 10.1016/j.immuni.2013.10.006
79. Bourgeois EA, Subramaniam S, Cheng T-Y, De Jong A, Layre E, Ly D, et al. Bee venom processes human skin lipids for presentation by CD1a. *J Exp Med*. (2015) 212:149–63. doi: 10.1084/jem.20141505
80. Jarrett R, Salio M, Lloyd-Lavery A, Subramaniam S, Bourgeois E, Archer C, et al. Filaggrin inhibits generation of CD1a neolipid antigens by house dust mite–derived phospholipase. *Sci Transl Med*. (2016) 8:325ra18. doi: 10.1126/scitranslmed.aad6833
81. Dudeck A, Dudeck J, Scholten J, Petzold A, Surianarayanan S, Köhler A, et al. Mast cells are key promoters of contact allergy that mediate the adjuvant effects of haptens. *Immunity*. (2011) 34:973–84. doi: 10.1016/j.immuni.2011.03.028
82. Dudeck J, Medyukhina A, Fröbel J, Svensson C-M, Kotrba J, Gerlach M, et al. Mast cells acquire MHCII from dendritic cells during skin inflammation. *J Exp Med*. (2017) 214:3791–811. doi: 10.1084/jem.20160783
83. Kato N, Nakanishi M, Hirashima N. Transbilayer asymmetry of phospholipids in the plasma membrane regulates exocytotic release in mast cells. *Biochemistry*. (2002) 41:8068–74. doi: 10.1021/bi016022v
84. Hammond GRV. Elimination of plasma membrane phosphatidylinositol (4,5)-bisphosphate is required for exocytosis from mast cells. *J Cell Sci*. (2006) 119:2084–94. doi: 10.1242/jcs.02912
85. Martin S, Pombo I, Poncet P, David B, Arock M, Blank U. Immunologic stimulation of mast cells leads to the reversible exposure of phosphatidylserine in the absence of apoptosis. *Int Arch Allergy Immunol*. (2000) 123:249–58. doi: 10.1159/000024451
86. Suzuki J, Fujii T, Imao T, Ishihara K, Kuba H, Nagata S. Calcium-dependent phospholipid scramblase activity of TMEM16 protein family members. *J Biol Chem*. (2013) 288:13305–16. doi: 10.1074/jbc.M113.457937
87. SmrŽ D, Dráberová L, Dráber P. Non-apoptotic phosphatidylserine externalization Induced by engagement of glycosylphosphatidylinositol-anchored proteins. *J Biol Chem*. (2007) 282:10487–97. doi: 10.1074/jbc.M611090200
88. Martin TW, Lagunoff D. Interactions of lysophospholipids and mast cells. *Nature*. (1979) 279:250–2. doi: 10.1038/279250a0
89. Nakahashi-Oda C, Tahara-Hanaoka S, Shoji M, Okoshi Y, Nakano-Yokomizo T, Ohkohchi N, et al. Apoptotic cells suppress mast cell inflammatory responses via the CD300a immunoreceptor. *J Exp Med*. (2012) 209:1493–503. doi: 10.1084/jem.20120096
90. Guo N, Her GR, Reinhold VN, Brennan MJ, Siraganian RP, Ginsburg V. Monoclonal antibody AA4, which inhibits binding of IgE to high affinity receptors on rat basophilic leukemia cells, binds to novel &-galactosyl derivativesof ganglioside GDlb. *J Biol Chem*. (1989)13267–72.
91. Mazucato VM, Silveira e Souza AMM, Nicoletti LM, Jamur MC, Oliver C. GD1b-derived gangliosides modulate FcεRI endocytosis in mast cells. *J Histochem Cytochem*. (2011) 59:428–40. doi: 10.1369/0022155411400868
92. Filho EGF, da Silva EZM, Zanotto CZ, Oliver C, Jamur MC. Cross-linking mast cell specific gangliosides stimulates the release of newly formed lipid mediators and newly synthesized cytokines. *Med Inflamm*. (2016) 2016:1–10. doi: 10.1155/2016/9160540
93. Pomorski TG, Menon AK. Lipid somersaults: uncovering the mechanisms of protein-mediated lipid flipping. *Progr Lipid Res*. (2016) 64:69–84. doi: 10.1016/j.plipres.2016.08.003
94. Saito K, Fujimura-Kamada K, Furuta N, Kato U, Umeda M, Tanaka K. Cdc50p, a protein required for polarized growth, associates with the Drs2p P-type ATPase implicated in phospholipid translocation in *saccharomyces cerevisiae*. *Mol Biol Cell*. (2004) 15:3418–32. doi: 10.1091/mbc.e03-11-0829
95. Folmer DE, Elferink RPJO, Paulusma CC. P4 ATPases - lipid flippases and their role in disease. *Biochim Biophys Acta*. (2009) 1791:628–35. doi: 10.1016/j.bbalip.2009.02.008
96. Baldridge RD, Graham TR. Identification of residues defining phospholipid flippase substrate specificity of type IV P-type ATPases. *Proc Natl Acad Sci USA*. (2012) 109:E290–8. doi: 10.1073/pnas.1115725109
97. Panatala R, Hennrich H, Holthuis JCM. Inner workings and biological impact of phospholipid flippases. *J Cell Sci*. (2015) 128:2021–32. doi: 10.1242/jcs.102715
98. Cole SPC. Multidrug resistance protein 1 (MRP1, ABCC1), a "multitasking" ATP-binding cassette (ABC) transporter. *J Biol Chem*. (2014) 289:30880–8. doi: 10.1074/jbc.R114.609248
99. Leier I, Jedlitschky G, Buchholz U, Cole SP, Deeley RG, Keppler D. The MRP gene encodes an ATP-dependent export pump for leukotriene C4 and structurally related conjugates. *J Biol Chem*. (1994) 269:27807–10. doi: 10.1097/00001813-199409001-00004
100. Evers R, Cnubben NH., Wijnholds J, van Deemter L, van Bladeren PJ, Borst P. Transport of glutathione prostaglandin A conjugates by the multidrug resistance protein 1. *FEBS Lett*. (1997) 419:112–6. doi: 10.1016/S0014-5793(97)01442-7
101. Brechbuhl HM, Min E, Kariya C, Frederick B, Raben D, Day BJ. Select cyclopentenone prostaglandins trigger glutathione efflux and the role of ABCG2 transport. *Free Radic Biol Med*. (2009) 47:722–30. doi: 10.1016/j.freeradbiomed.2009.06.005
102. Mitra P, Oskeritzian CA, Payne SG, Beaven MA, Milstien S, Spiegel S. Role of ABCC1 in export of sphingosine-1-phosphate from mast cells. *Proc Natl Acad Sci USA*. (2006) 103:16394–9. doi: 10.1073/pnas.0603734103
103. Quazi F, Molday RS. Lipid transport by mammalian ABC proteins. *Essays Biochem*. (2011) 50:265–90. doi: 10.1042/bse0500265
104. Sahu SK, Gummadi SN, Manoj N, Aradhyam GK. Phospholipid scramblases: an overview. *Arch Biochem Biophys*. (2007) 462:103–14. doi: 10.1016/j.abb.2007.04.002
105. Amir-Moazami O, Alexia C, Charles N, Launay P, Monteiro RC, Benhamou M. Phospholipid scramblase 1 modulates a selected set of IgE receptor-mediated mast cell responses through LAT-dependent pathway. *J Biol Chem*. (2008) 283:25514–23. doi: 10.1074/jbc.M705320200
106. Zhou Q, Zhao J, Stout JG, Luhm RA, Wiedmer T, Sims PJ. Molecular cloning of human plasma membrane phospholipid scramblase: a protein mediating transbilayer movement of plasma membrane phospholipids. *J Biol Chem*. (1997) 272:18240–4. doi: 10.1074/jbc.272.29.18240
107. Zhou Q. Normal hemostasis but defective hematopoietic response to growth factors in mice deficient in phospholipid scramblase 1. *Blood*. (2002) 99:4030–8. doi: 10.1182/blood-2001-12-0271
108. Wiedmer T, Zhao J, Nanjundan M, Sims PJ. Palmitoylation of phospholipid scramblase 1 controls its distribution between nucleus and plasma membrane. *Biochemistry*. (2003) 42:1227–33. doi: 10.1021/bi026679w
109. Gilfillan AM, Tkaczyk C. Integrated signalling pathways for mast-cell activation. *Nat Rev Immunol*. (2006) 6:218–30. doi: 10.1038/nri1782
110. Cui W, Li S-Y, Du J-F, Zhu Z-M, An P. Silencing phospholipid scramblase 1 expression by RNA interference in colorectal cancer and metastatic liver cancer. *Hepatobil Pancr Dis Int*. (2012) 11:393–400. doi: 10.1016/S1499-3872(12)60197-0
111. Kantari C, Pederzoli-Ribeil M, Amir-Moazami O, Gausson-Dorey V, Moura IC, Lecomte M-C, et al. Proteinase 3, the Wegener

autoantigen, is externalized during neutrophil apoptosis: evidence for a functional association with phospholipid scramblase 1 and interference with macrophage phagocytosis. *Blood*. (2007) 110:4086–95. doi: 10.1182/blood-2007-03-080457
112. Huang Y, Zhao Q, Zhou C-X, Gu Z-M, Li D, Xu H-Z, et al. Antileukemic roles of human phospholipid scramblase 1 gene, evidence from inducible PLSCR1-expressing leukemic cells. *Oncogene*. (2006) 25:6618–27. doi: 10.1038/sj.onc.1209677
113. Ben-Efraim I, Zhou Q, Wiedmer T, Gerace L, Sims PJ. Phospholipid scramblase 1 is imported into the nucleus by a receptor-mediated pathway and interacts with DNA. *Biochemistry*. (2004) 43:3518–26. doi: 10.1021/bi0356911
114. Zhou Q, Ben-Efraim I, Bigcas J-L, Junqueira D, Wiedmer T, Sims PJ. Phospholipid scramblase 1 binds to the promoter region of the inositol 1,4,5-triphosphate receptor type 1 gene to enhance its expression. *J Biol Chem*. (2005) 280:35062–8. doi: 10.1074/jbc.M504821200
115. Sun J, Nanjundan M, Pike LJ, Wiedmer T, Sims PJ. Plasma membrane phospholipid scramblase 1 is enriched in lipid rafts and interacts with the epidermal growth factor receptor. *Biochemistry*. (2002) 41:6338–45. doi: 10.1021/bi025610l
116. Nanjundan M, Sun J, Zhao J, Zhou Q, Sims PJ, Wiedmer T. Plasma membrane phospholipid scramblase 1 promotes EGF-dependent activation of c-Src through the epidermal growth factor receptor. *J Biol Chem*. (2003) 278:37413–8. doi: 10.1074/jbc.M306182200
117. Pastorelli C, Veiga J, Charles N, Voignier E, Moussu H, Monteiro RC, et al. Phospholipid scramblase, a new effector of FcεRI signaling in mast cells. *Mol Immunol*. (2002) 38:1235–8. doi: 10.1016/S0161-5890(02)00069-X
118. Kassas A, Moura IC, Yamashita Y, Scheffel J, Guérin-Marchand C, Blank U, et al. Regulation of the tyrosine phosphorylation of phospholipid scramblase 1 in mast cells that are stimulated through the high-affinity IgE receptor. *PLoS ONE*. (2014) 9:e109800. doi: 10.1371/journal.pone.0109800
119. Kassas-Guediri A, Coudrat J, Pacreau E, Launay P, Monteiro RC, Blank U, et al. Phospholipid scramblase 1 amplifies anaphylactic reactions *in vivo*. *PLoS ONE*. (2017) 12:e0173815. doi: 10.1371/journal.pone.0173815
120. Dichlberger A, Kovanen PT, Schneider WJ. Mast cells: from lipid droplets to lipid mediators. *Clini Sci*. (2013) 125:121–30. doi: 10.1042/CS20120602
121. Talukder AH, Bao M, Kim TW, Facchinetti V, Hanabuchi S, Bover L, et al. Phospholipid Scramblase 1 regulates Toll-like receptor 9-mediated type I interferon production in plasmacytoid dendritic cells. *Cell Res*. (2012) 22:1129–39. doi: 10.1038/cr.2012.45
122. Tsai M-H, Lee C-K. STAT3 cooperates with phospholipid scramblase 2 to suppress type I interferon response. *Front Immunol*. (2018) 9:1886. doi: 10.3389/fimmu.2018.01886
123. Mutch DM, O'Maille G, Wikoff WR, Wiedmer T, Sims PJ, Siuzdak G. Mobilization of pro-inflammatory lipids in obese Plscr3-deficient mice. *Genome Biol*. (2007) 8:R38. doi: 10.1186/gb-2007-8-3-r38

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The handling Editor declared a shared affiliation, though no other collaboration, with the authors PH and ZO.

frontiers in Immunology

ORIGINAL RESEARCH
published: 18 September 2019
doi: 10.3389/fimmu.2019.02177

1-Palmitoyl-2-Linoleoyl-3-Acetyl-rac-Glycerol (PLAG) Rapidly Resolves LPS-Induced Acute Lung Injury Through the Effective Control of Neutrophil Recruitment

Ha-Reum Lee[1†], *Su-Hyun Shin*[2,3†], *Joo Heon Kim*[4], *Ki-Young Sohn*[1], *Sun Young Yoon*[1] *and Jae Wha Kim*[2,3*]

[1] ENZYCHEM Lifesciences, Seoul, South Korea, [2] Division of Systems Biology and Bioengineering, Cell Factory Research Center, Korea Research Institute of Bioscience and Biotechnology, Daejeon, South Korea, [3] Department of Functional Genomics, University of Science and Technology, Daejeon, South Korea, [4] Department of Pathology, EulJi University School of Medicine, Daejeon, South Korea

OPEN ACCESS

Edited by:
Katarzyna Anna Duda,
Forschungszentrum Borstel (LG), Germany

Reviewed by:
Mieke Gouwy,
KU Leuven, Belgium
Juerg Hamacher,
Lindenhofspital, Switzerland

***Correspondence:**
Jae Wha Kim
wjkim@kribb.re.kr

[†]These authors have contributed equally to this work as first authors

Specialty section:
This article was submitted to Inflammation, a section of the journal Frontiers in Immunology

Received: 18 July 2018
Accepted: 28 August 2019
Published: 18 September 2019

Citation:
Lee H-R, Shin S-H, Kim JH, Sohn K-Y, Yoon SY and Kim JW (2019) 1-Palmitoyl-2-Linoleoyl-3-Acetyl-rac-Glycerol (PLAG) Rapidly Resolves LPS-Induced Acute Lung Injury Through the Effective Control of Neutrophil Recruitment. Front. Immunol. 10:2177. doi: 10.3389/fimmu.2019.02177

Acute lung injury (ALI) is an acute respiratory failure that is associated with excessive neutrophil recruitment and high mortality. To assess the efficacy of 1-palmitoyl-2-linoleoyl-3-acetyl-rac-glycerol (PLAG) as a therapeutic agent for ALI, this compound was administered orally to mice challenged with an intranasal dose of lipopolysaccharide (LPS). Using this model, we found that PLAG promotes resolution of ALI through effective control of LPS-induced neutrophil infiltration, endothelial permeability, and inflammatory chemokine production. In addition, the Toll like Receptor 4 (TLR4) endocytosis/exocytosis cycle was significantly accelerated in Raw 264.7 cells co-treated with PLAG/LPS, as compared to cells treated only with LPS. During this cycle, a PLAG-induced exotoxin clearance pathway was observed to occur through the prompt assembly of nicotinamide adenine dinucleotide phosphate (NADPH) units and production of reactive oxygen species (ROS), which ultimately lead to earlier LPS clearance. We further detected reduced expression, as well as faster return to homeostatic levels, of macrophage inflammatory protein (MIP)-2, in PLAG/LPS- vs. LPS-treated cells. MIP-2 is a main inducer of neutrophil migration that is mainly controlled by interferon regulatory factor 3 (IRF3) activation and is involved in the TLR4 endosomal-signaling pathway. PLAG induced TLR4-mediated TRIF-related adaptor molecules/Toll-interleukin receptor (TIR) domain-containing adaptor protein including interferon (IFN)-β/IRF3 endosomal signaling, leading to rapid association of TRAM/TRIF and TLR4 and earlier IRF3 phosphorylation in PLAG/LPS-treated vs. LPS-treated cells. PLAG specificity was further verified with PLAG analogs and metabolites known to control excessive neutrophil infiltration, suggesting that this acetylated diacylglycerol has a unique biological role in neutrophil motility. Thus, our data indicate that PLAG may represent a potential therapeutic agent for resolution of LPS-induced lung inflammation through effective MIP-2 modulation.

Keywords: acute lung injury, neutrophil, transmigration, inflammation, PLAG, resolution

INTRODUCTION

Acute lung injury (ALI) is a severe inflammatory lung disease that is characterized by the disruption of the lung alveolar-capillary membrane barrier. This leads to a massive infiltration of neutrophils into the interstitium and the bronchoalveolar space, as well as an excessive inflammatory response (1). The activation and transmigration of neutrophils are considered an essential step in ALI progression. Neutrophils play a key role in the innate immune system, as these cells are the first leukocytes to migrate to regions of acute inflammation (2). Neutrophils cross the blood vessel endothelium into infected tissue and eliminate invading pathogens via multiple killing mechanisms, including phagocytosis, degranulation, and neutrophil extracellular traps (NETs) (3, 4). Notably, neutrophils secrete numerous cytokines and chemokines that influence other immune cells and are thus key regulators of inflammation (5). Although the transmigration and activation of neutrophils are absolutely essential for infection clearance, excessive recruitment, and aberrant activation of neutrophils leads to severe host tissue damage and may ultimately cause death (6).

Lipopolysaccharide (LPS) is a Gram-negative bacterial toxin recognized by Toll-like receptor 4 (TLR4), which is used to induce both acute and chronic tissue injury in animal models of inflammation (7, 8). When LPS infiltrates into the lung, macrophages are activated by the LPS-receptor complex, TLR4/MD-2, and this stimulates the translocation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) from the cytosol to membrane (9). Activated NOX produces reactive oxygen species (ROS) that are able to directly kill pathogens and stimulate increased expression of interleukin (IL)-8 and macrophage inflammatory protein (MIP)-2 (10). Neutrophil migration is regulated by the response to chemokine gradients, and MIP-2, in particular, is a major chemokine involved in the induction of neutrophil migration (11, 12).

TLR4-associated signaling pathways are classified based on the involvement of two main adaptor proteins, referred to as myeloid differentiation primary response protein 88 (Myd88) and Toll-interleukin receptor (TIR) domain-containing adaptor protein inducing interferon (IFN)-β (TRIF) (13). The Myd88-dependent pathway, which occurs mainly at the plasma membrane, is mediated by Myd88 and the TIR domain-containing adapter protein (TIRAP). This pathway activates nuclear transcription factor kappa B (NF-κB) signaling and leads to induction IL-1β and tumor necrosis factor (TNF) (14). In contrast, the TRIF-dependent pathway utilizes the TRIF-related adaptor molecules (TRAM) and TRIF and is associated with endocytosis of the activated TLR4 receptor. This occurs at the endosomal membrane following TLR4 internalization and activates signaling via the interferon regulatory factor 3 (IRF3) transcription factor, as well as IFN-β secretion (15). Critically, the TLR4-mediated signaling pathways play crucial roles in the immune response and in host defense by acting as sensors of microbial infection.

The acetylated diacylglycerol 1-Palmitoyl-2-linoleoyl-3-acetyl-rac-glycerol (PLAG), is a mono-acetyl-diglyceride that has been isolated from the antlers of sika deer and can be chemically synthesized from glycerol, palmitic acid, and linoleic acid (16). Synthesized PLAG is chemically identical to its naturally isolated form (17). In a previous study, we found that PLAG exerts therapeutic effects when administered in combination pegfilgrastim for treatment of chemotherapy-induced neutropenia. PLAG modulated neutrophil transmigration, and significantly reduced fluorouracil/scratching-induced oral mucositis and cachexia (18, 19). Here, we investigated the effects of PLAG on neutrophil migration and the resolution of inflammation in an LPS-induced mouse model of ALI and on TLR4 signaling of macrophage cells in culture. We show that PLAG modulates TLR4 endocytosis-dependent endosomal signaling, TRAM-TRIF-IRF3-mediated signaling, and ultimately can control excessive neutrophil infiltration through modulation of MIP-2 expression. These findings suggest that PLAG may have therapeutic potential for treatment of ALI and other severe inflammatory diseases.

MATERIALS AND METHODS

LPS-Induced ALI Mouse Model

Balb/c mice (9-week to 11-week-old males) were purchased from Koatech Co. (Pyongtaek, Republic of Korea) and maintained under specific pathogen-free (SPF) conditions. For the ALI model, mice were anesthetized with 2,2,2-Tribromoethanol (150 mg/kg; Sigma-Aldrich, St. Louis, MO, USA) by intraperitoneal injection and administered LPS intranasally (25 mg/kg; Sigma-Aldrich). PLAG (10, 50, or 250 mg/kg, Enzychem Lifesciences Co., Daejeon, Republic of Korea) was administered orally. Collection of bronchoalveolar lavage fluid (BALF) was performed by tracheal cannulation, using cold phosphate-buffered saline (PBS). Complete blood counts (CBCs) were performed using the Mindray BC-5300 Auto Hematology Analyzer (Shenzhen Mindray Bio-medical Electronics, China).

Ethics Statement

All animal experimental procedures were performed in accordance with the Guide and Use of Laboratory Animals (Institute of Laboratory Animal Resources). All experiments were approved by the Institutional Review Committee for Animal Care and Use of KRIBB (Korea Research Institute of Bioscience and Biotechnology, Daejeon, Republic of Korea), approval number KRIBB-AEC-16031.

Cell Culture and Primary Neutrophil Isolation

Raw 264.7 (American Type Culture Collection [ATCC], Manassas, VA, USA) cells were cultured in Dulbecco's Modified Eagle Medium, supplemented with 2 mM L-glutamine and 10% fetal bovine serum (FBS). Human Umbilical Vein Endothelial Cells (HUVEC; Lonza, Walkensville, MD, USA) were cultured in EGM^{TM}-2 media (Lonza) and BulletKit^{TM} supplement (Lonza). HL-60 (ATCC) cells were cultured in Roswell Park Memorial Institute (RPMI) 1640, containing 20% FBS (TCB, Long Beach, CA, USA) and differentiated with complete media, containing 1.5% dimethyl sulfoxide (DMSO) for 5 days. THP-1 (ATCC) cells were cultured in RPMI 1640, containing 10% FBS. All cells were maintained in a 5% CO_2 incubator at 37°C. Primary neutrophils were isolated from femurs and tibias of mice using 65 and 72% Percoll® (Sigma-Aldrich) two-layer gradients. After

centrifugation, recovered cells were incubated with red blood cell lysis buffer (ACK Lysing Buffer, Gibco, Waltham, MA, USA). The purity of isolated primary neutrophils ($CD11b^+GR1^+$) was determined to be >90% (data not shown).

Evans Blue Leakage Assay

Evans blue (50 mg/kg, Sigma-Aldrich) was diluted in PBS and injected intravenously into mice 30 min before sacrifice. After sacrifice, mice were perfused by right ventricle puncture with PBS, and lungs were photographed. Following drying at 56°C for 48 h, lungs were weighed, and Evans blue dye was extracted in 500 μl of formamide (Sigma-Aldrich). The absorbance of these supernatants was measured by spectrophotometry (Molecular Devices, Sunnyvale, CA, USA) at a wavelength of 620 nm. Evans blue concentrations were calculated as extracted Evans blue concentration (ng) divided by the dry lung tissue weight (mg) and compared to measurements from a standard curve.

Hematoxylin and Eosin Staining and Immunohistochemistry

Lung tissue specimens were fixed in 10% buffered formalin for 24 h, embedded in paraffin, and sectioned at 4 μm. Tissue sections were stained with hematoxylin and eosin (H&E). For immunohistochemistry (IHC) analyses, 4-μm thick lung serial sections were cut and mounted on charged glass slides (Superfrost Plus; Fisher Scientific, Rochester, NY, USA). The sections were deparaffinized and then treated with 3% hydrogen peroxide in methanol to quench the endogenous peroxidase activity. Samples were then incubated with 1% bovine serum albumin (BSA; Gibco) to block non-specific binding. After blocking, sections were incubated with primary rat anti-neutrophil (NIMP-R14, Thermo Fisher Scientific Inc., Waltham, MA, USA) antibody (1:100) or mouse anti-LPS (Abcam, Cambridge, UK) antibody (1:100) at 4°C overnight. After washing, the slides were incubated with a 1:250 dilution of secondary antibody, either horseradish peroxidase-conjugated goat-anti-rat IgG (Santa Cruz Biotechnology, Dallas, TX, USA) or horseradish peroxidase-conjugated goat-anti-mouse IgG (Dako, Santa Clara, CA, USA), at room temperature for 15 min. Images were observed under light microscopy (Olympus, Shinjuku, Tokyo, Japan).

Histological Scoring and Myeloperoxidase Activity Assay

Lung injury scores were measured by a blinded investigator using published criteria (**Table 1** and Equation 1), which are based on neutrophil infiltration (in the alveolar or the interstitial space), hyaline membranes, proteinaceous debris filling the airspaces, and septal thickening (20). To measure myeloperoxidase (MPO) activity in ALI mice, lungs were isolated and homogenized with 0.1% IGEPAL® CA-630 (Sigma-Aldrich). After centrifugation for 30 min, MPO activity was determined using the Myeloperoxidase Activity Assay Kit (Abcam). Sample absorbance was measured using a microplate reader (Molecular Devices) at 410 nm.

TABLE 1 | Lung injury scoring criteria from Matute-bello et al. (20).

Parameter	Score per field		
	0	1	2
A. Neutrophils in the alveolar space	None	1–5	>5
B. Neutrophils in the interstitial space	None	1–5	>5
C. Hyaline membranes	None	1	>1
D. Proteinaceous debris filling the airspaces	None	1	>1
E. Alveolar septal thickening	<2x	2x−4x	>4x

$$\text{The final score} = \frac{[(20 \times A) + (14 \times B) + (7 \times C) + (7 \times D) + (2 \times E)]}{\text{Number of fields} \times 100} \quad (1)$$

Reverse Transcription Polymerase Chain Reaction (RT-PCR) and Real-Time PCR

Total RNA was extracted using Total RNA Extraction Solution (Favorgen, Taiwan), according to the manufacturer's instructions. This RNA was used in reverse transcription reactions with oligo-dT primers and M-MLV RT reagents (Promega, Madison, WI, USA), according to the manufacturer's instructions. For RT-PCR, the synthesized cDNA was mixed with 2x PCR Master Mix (Solgent, Daejeon, Republic of Korea) and 10 pmol specific PCR primer pair following the manufacturer's protocol. The primers were synthesized from Macrogen (Seoul, Republic of Korea; see **Table 2** for primer sequences). Amplified products were separated on 1% agarose gels, stained with ethidium bromide, and photographed under UV illumination using a GelDoc (Bio-Rad Laboratories, Hercules, CA, USA).

A SYBR Green kit (Applied Biosystems, Foster City, CA, USA) was used for real-time PCR (qPCR) analysis of cDNA according to the manufacturer's instructions. Thermal cycling conditions were as follows: initial denaturation at 95°C for 15 min, followed by 40 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. A melting step was performed by raising the temperature from 72 to 95°C after the last cycle. Thermal cycling was conducted on a ViiA 7 Real-Time PCR System machine (Applied Biosystems). The target gene expression levels are shown as a ratio in comparison with GAPDH expression in the same sample by calculation of cycle threshold (Ct) value. The relative expression levels of target genes were calculated by the $2^{-\Delta\Delta CT}$ relative quantification method.

Enzyme-Linked Immunosorbent Assay (ELISA)

Concentrations of MIP-2, IFN-β, IL-1β, and TNF were measured using ELISA kits for MIP-2 (R&D Systems, Minneapolis, MN, USA), mouse IFN-β (R&D Systems), mouse IL-1β (BD Biosciences, Franklin Lakes, NJ, USA), and mouse TNF (BD Biosciences), according to the manufacturers' instructions. Cytokine levels were estimated by interpolation from a standard curve generated using an ELISA reader (Molecular Devices) at 450 nm.

TABLE 2 | Primers used for PCR.

	Sense primer	Antisense primer
MIP-2	AGT GAA CTG CGC TGT CAA TG	CTT TGG TTC TTC CGT TGA GG
S100A8	ATG CCG TCT GAA CTG GAG AA	TGC TAC TCC TTG TGG CTG TC
S100A9	ATG GCC AAC AAA GCA CC TT	TTA CTT CCC ACA GCC TTT GC
GAPDH	CCA TCA CCA TCT TCC AGG AG	ACA GTC TTC TGG GTG GCA GT
TRIF	TGT TGG AAA GCA GTG GCC TAT	GAT GAC GTG GTG TTC TGC AGA
TRAM	AGG CTA CAC AGA GAA ACC CC	TGT GAC TTC CTG GCC ATG AT
TIRAP	GAT CGT CAC CAG CTT CCA TT	CCT GAT GCC AGA GGA AGA AG
Myd88	TCG AGT TTG TGC AGG AGA TG	AGG CTG AGT GCA AAC TTG GT
IFNβ	AAG AGT TAC ACT GCC TTT GCC ATC	CAC TGT CTG CTG GTG GAG TTC ATC
IL-1β	TGT AAT GAA AGA CGG CAC ACC	TCT TCT TTG GGT ATT GCT TGG
TNF	ATG AGA AGT TCC CAA ATG GC	CTC CAC TTG GTG GTT TGC TA
LPL	GGG CTC TGC CTG AGT TGT AG	GTC AGG CCA GCT GAA GTA GG
GPI-HBP1	AGC AGG GAC AGA GCA CCT CT	AGA CGA GCG TGA TGC AGA AG
Clathrin	GGG CAA ATC AAA GAA GTG GA	GAG CAG TCA ACA TCC AGC AA
Caveolin-1	ACC TCT CTG GAC TGG CAG AA	GGA AAG GTC GAG CTT CAC AG
IL-6	GAT GCT ACC AAA CTG GAT AT	GGT CCT TAG CCA CTC CTT CTG TG
CXCL1	AGA CTG CTC TGA TGG CAC CT	CTG CAC TTC TTT TCG CAC AA
CXCL3	CAA CGG TGT CTG GAT GTG TC	AGC CAA GGA ATA CTG CCT TA
CXCL5	GTA TCC TGG GTT TCC GGA CT	GAT CTC CAT CGC TTT CTT CG
CXCL12	GAG CCA ACG TCA AGC ATC TG	CGG GTC AAT GCA CAC TTG TC
CCL2	CCC AAT GAG TAG GCT GGA GA	AAA ATG GAT CCA CAC CTT GC
CCL3	CCA AGT CTT CTC AGC GCC AT	TCC GGC TGT AGG AGA AGC AG
CCL4	TCT TGC TCG TGG CTG CCT	GGG AGG GTC AGA GCC CA
CCL5	ATA TGG CTC GGA CAC CAC TC	AGC AAG CAA TGA CAG GGA AG
CCL7	GTG TCC CTG GGA AGC TGT TA	TCC TTA GGC GTG ACC ATT TC
CCR1	AAG GCC CAG AAA CAA AGT CT	TCT GTA GTT GTG GGG TAG GC
CCR2	CCT GCA AAG ACC AGA AGA GG	GTG AGC AGG AAG AGC AGG TC
CCR3	AAG GAC TTA GCA AAA TTC AC CA	ACA CCA GGG AGT ACA GTG GA
CCR5	CGT TCC CCC TAC AAG AGA CT	ACC CAC AAA ACC AAA GAT GA
CXCR1	TCA GTG GTT CCT GCT GC TG	GCA GAC GAG GAT AGT GAG CA
CXCR2	GAT GTC TAC CTG CTG AAC CT	ACC AGG TTG TAG GGC AGC CA
CXCR4	GGG GAC ATC AGT CA GG	GTG GAA GAA GGC GAG GG

Immunofluorescence Staining and Flow Cytometric Analysis

To detect TLR4/MD2 on membrane surfaces, cells were fixed with 2% paraformaldehyde (Sigma-Aldrich) and blocked with PBS, containing 1% BSA (Gibco). Cells were then incubated with rabbit anti-TLR4/MD2 antibody (Thermo Fisher Scientific Inc.) and Alexa488-conjugated anti-rabbit IgG (Invitrogen, Carlsbad, CA, USA) without permeabilization. For detection of p47phox, Rac-1, lysosomes, ROS, and intracellular LPS, cells were fixed and permeabilized. Samples were then stained with rabbit anti-p47phox (Thermo Fisher Scientific Inc.), mouse anti-Rac-1 (Merck Millipore, Billerica, MA, USA), FITC-conjugated CM-H2DCFDA (Invitrogen) for ROS, the Texas red-conjugated LYSO-ID® Red Detection Kit (Enzo Life Sciences, Inc.), or mouse anti-LPS (Abcam) antibodies, respectively. Secondary antibody staining was then performed with Alexa488-conjugated anti-rabbit IgG (Invitrogen) or Alexa594-conjugated anti-mouse IgG (Thermo Fisher Scientific Inc.). For confocal microscopy analysis, cells were washed with PBS and mounted in 4',6-diamidino-2-phenylindole (DAPI)-containing fluorescence microscopy mounting medium (Invitrogen). Samples were analyzed with a laser scanning confocal microscope (Carl Zeiss, Oberkochen, Germany). For flow cytometric analysis, cells were washed with PBS and analyzed with a FACSVerse Flow Cytometer (BD Biosciences), and the data were processed with FlowJo software (Tree Star, OR, USA).

siRNA Transfection

Specific siRNA targeting TRIF (sc-154266), TRAM (sc-44748), TIRAP (sc-44740), Myd88 (sc-35987), lipoprotein lipase (LPL) (sc-44900), glycosylphosphatidylinositol high density binding protein 1 (GPI-HBP1) (sc-145686), clathrin (sc-35067), and caveolin-1 (sc-29241) were purchased from Santa Cruz Biotechnology. Scramble siRNA (sc-37007) used as control. Cells were transfected with the indicated siRNA duplex targeting constructs (50 nM) and HiPerFect Transfection Reagent (QIAGEN, Hilden, Germany). After incubation for 24 h, downregulation of target gene expression was evaluated by RT-PCR.

Immunoprecipitation

For immunoprecipitations, cells were extracted in lysis buffer containing 25 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% NP-40, and 5% glycerol with protease inhibitor (Roche, Indianapolis, IN, USA). Anti-TLR4/MD2 antibody (Thermo Fisher Scientific Inc.) (2 μg) was added to 200 μl of each cellular extract, and these were incubated at 4°C for 6 h on a rotator. Pre-washed protein G beads (Bio-Rad) (100 μl) were then added to each sample, followed by incubation at 4°C for 4 h. Samples were washed three times in PBS-T [PBS, containing 0.05% Tween-20 (Merck Millipore, Billerica, MA, USA)], solubilized in Laemmli buffer at 70°C for 10 min, boiled for 10 min, and verified by western blot.

Western Blot Analysis

Cells were ruptured on ice with 1x RIPA lysis buffer (Cell Signaling Technology, Danvers, MA, USA), containing protease inhibitor (Roche) and phosphatase inhibitor (Thermo Fisher Scientific Inc.). Cell lysates were clarified by centrifugation, and samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins in gels were transferred onto polyvinylidine (PVDF) membranes (Bio-Rad), and these were blocked with 5% BSA (Gibco) in PBS-T. Membranes were then incubated with antibodies against TRIF (Abcam), Myd88 (Cell Signaling Technology), TLR4 (Invitrogen), phospho-TRAM (MyBioSource, San Diego, CA, USA), TRAM (R&D Systems), phospho-TIRAP (Y86; Abcam), TIRAP (Abcam), phospho-IRF3 (Ser396; Cell Signaling Technology), IRF3 (Cell Signaling Technology), phospho-p65 (Ser536; Cell Signaling Technology), and p65 (Enzo Life Sciences, Inc.) overnight at 4°C. After washing with PBS-T, membranes were stained with peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology) or peroxidase-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology). Target proteins were then detected using the Immobilon Western Chemiluminescent HRP Substrate (Merck Millipore).

In vitro Permeability Assay and Cell Migration Assay

To measure the permeability of endothelial cells *in vitro*, HUVEC cells (1×10^4) were seeded onto 5-μm pore Transwell inserts (Corning Inc., Corning, NY, USA) in complete media. After 16 h, supernatants were removed, and 5×10^4 differentiated HL-60 (dHL-60) cells were loaded onto the HUVEC cells into the top Transwell chamber. THP-1 cells were pre-incubated with PLAG (100 μg/ml) for 1 h and then stimulated with LPS (100 ng/ml). After 16 h, these were centrifuged, and the supernatant was transferred into the bottom Transwell chambers. The combined Transwells were incubated at 37°C for 6 h, after which migrated dHL-60 cells were counted using a hemocytometer and trypan blue staining. Transwell assays were also used to verify albumin efflux into the alveolar compartment observed in the *in vivo* animal model. Briefly, in place of albumin, streptavidin-HRP was laid on the upper chamber with HL-60 cells for 5 min, and then the medium (100 μl) containing transmigrated HRP in the lower chamber was collected and assayed for activity using 100 μl of 3,3',5,5'-tetramethylbenzidine (TMB) substrate (Surmodics, Eden Prairie, MN, USA). Color development was measured by microplate reader (Molecular Devices) at 450 nm.

To measure migration of primary neutrophils, Raw264.7 cells were pre-incubated with PLAG (100 μg/ml) for 1 h and stimulated with LPS (100 ng/ml) for 16 h. Cells were centrifuged, and the supernatant was transferred to the bottom chamber of a Transwell plate. Isolated mouse neutrophils were suspended in RPMI 1640 without FBS, and loaded onto 3 μm-pore Transwell filters (Corning) positioned on top of the migration chamber. Combined Transwells were incubated at 37°C for 2 h, and migrated neutrophils were counted using a hemocytometer with trypan blue staining.

Statistical Analysis

Results are presented as the mean ± standard error of the mean (s.e.m.). The level of significance, assumed at the 95% confidence limit or greater ($p < 0.05$), was calculated with one-way analysis of variance (ANOVA), followed by Duncan's *post hoc* test, using SPSS software; * indicates $p < 0.05$.

RESULTS

PLAG Resolves LPS-Induced ALI Through Regulation of Excessive Neutrophil Infiltration

LPS is able to recruit immune cells into the lung alveolar compartment and promotes the secretion of inflammatory mediators. Thus, it is commonly used to induce development of ALI in a mouse model (21). Evans blue dye extravasation into the tissue can further used as an index of increased vascular permeability and neutrophil transmigration in ALI and control mice (22). Here, we used this Evans blue leakage assay to investigate the effects of PLAG (administered orally) on vascular leakage in mice that were intranasally administered with LPS. We found that in mice treated with LPS alone for 16 h, lung tissues showed excessive leakage of albumin from blood vessels to the alveolar space, as demonstrated by increased Evans blue staining (**Figure 1A**). Lungs from mice co-treated with PLAG/LPS, however, showed decreased Evans blue-stained albumin. These findings were confirmed by a quantitative analysis of Evans blue-labeled albumin extract from the lungs (**Supplementary Figure 1a**), which shows a decreased level of Evans blue dye in lungs from mice treated with PLAG/LPS, as compared to LPS alone.

A high level of Evans blue staining is correlated with the vast extravasation of neutrophils into the alveolar space. We therefore, examined the effect of PLAG on leukocyte cell infiltration into the lung alveolar compartment using H&E staining. These data revealed that intranasal LPS administration induces extensive inflammatory cell infiltration into the lung tissue compared to control animals. However, PLAG/LPS co-treated mice exhibit a considerably reduced inflammatory cell infiltration into the alveolar space and display normal alveolar morphology (**Figure 1B**). Histological scores of control, LPS, and PLAG/LPS-treated mice show the same effect (**Figure 1C**, **Table 1**, and Equation 1).

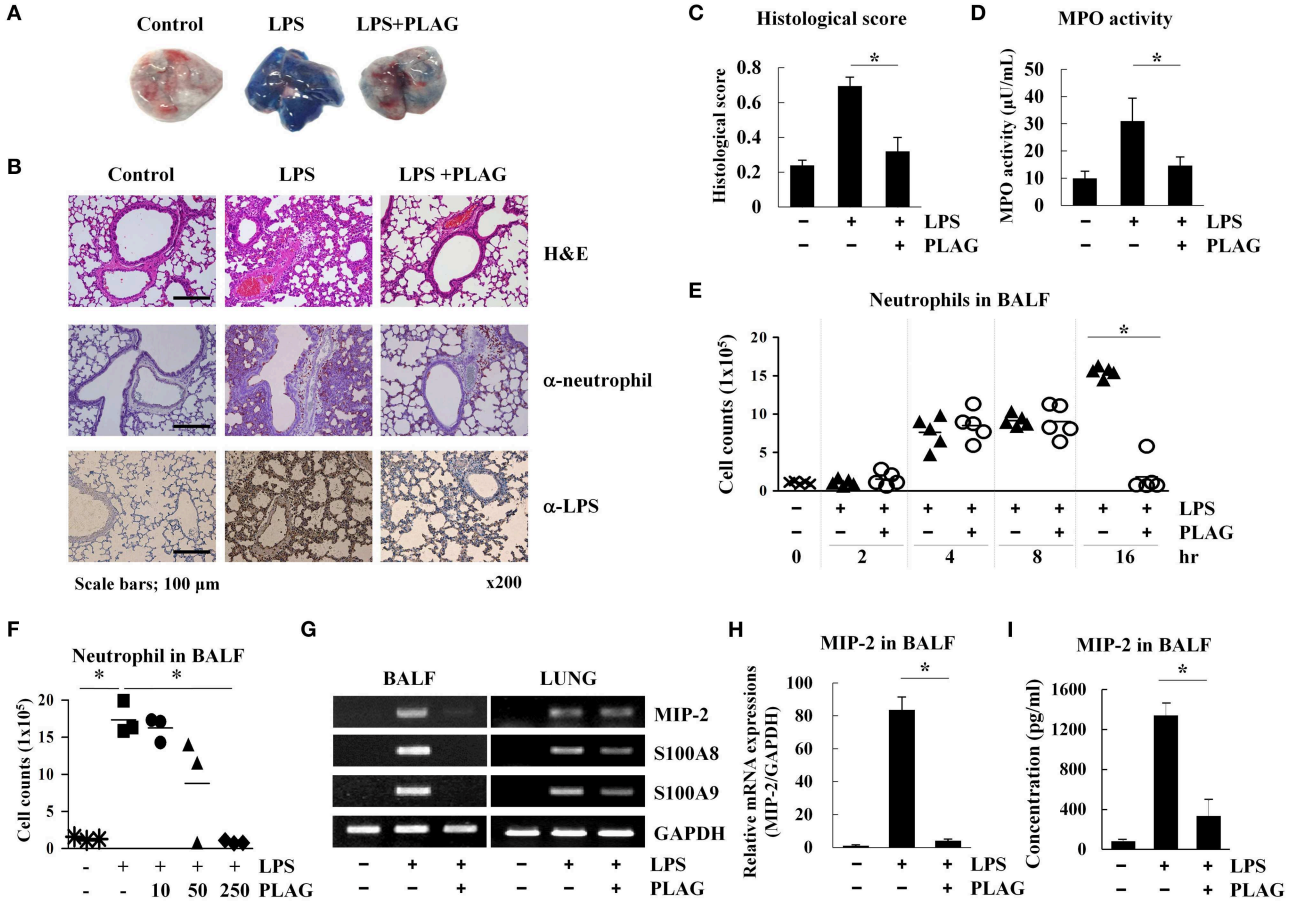


FIGURE 1 | PLAG suppresses lung inflammation in a mouse model of acute lung injury (ALI) through regulation of neutrophil infiltration. Mice were divided into four separate groups ($n = 5$ per group): control, LPS-treated, and PLAG/LPS co-treated. LPS (25 mg/kg) was intranasally injected, and PLAG (250 mg/kg) was administered orally. Evans blue dye (50 mg/kg) was injected intravenously 30 min before sacrifice following LPS or PLAG/LPS treatment for 16 h, and lungs were harvested from all animals. Representative lungs demonstrating Evans blue accumulation are shown **(A)**. Histological examination of lung tissues was performed 16 h after LPS administration. Lung sections were stained with HandE or with neutrophil and LPS-specific antibodies **(B)**. Lung injury scoring was calculated as described in **Table 1** and Equation 1 **(C)** (20). Lungs from LPS and PLAG/LPS-treated animals, as well as controls, were examined for MPO activity **(D)**. Following LPS or PLAG/LPS stimulation for 2, 4, 8, and 16 h, mice were sacrificed, and the number of neutrophils in bronchoalveolar lavage fluid (BALF) was counted using complete blood count (CBC) analysis **(E)**. The bar represents the mean. Mice were divided into five separate groups ($n = 3$ per group): control, LPS-treated, PLAG (10, 50, 250 mg/kg)/LPS co-treated. LPS (25 mg/kg) was intranasally injected and PLAG was administered orally. Following LPS for 16 h, mice were sacrificed, and the number of neutrophils in bronchoalveolar lavage fluid were counted using complete blood count (CBC) analysis **(F)**.Total RNA was extracted from BALF cells and homogenized lungs and analyzed by reverse transcription (RT)-PCR **(G)** and real-time PCR (qPCR) **(H)**. GAPDH was used as a control. Levels of secreted MIP-2 in BALF were measured using an ELISA assay **(I)**. All *in vivo* data were obtained from at least three independent experiments with five mice for each group **(A–G)**. Data shown represent one experiment performed in triplicate. *indicates $p < 0.05$.

An increase in MPO activity reflects neutrophil accumulation in the lungs. Here, we found that MPO activity of isolated lung tissue is substantially increased in LPS-treated mice but is significantly decreased in the PLAG/LPS co-treated mice, as compared to those treated with LPS alone (**Figure 1D**). These data suggest that PLAG plays a protective role in ALI by blocking excessive neutrophil influx into the lung tissue. To further test this hypothesis, neutrophils in BALF were counted 2, 4, 8, and 16 h after LPS intranasal administration (**Figure 1E**). We found that LPS challenge significantly increases neutrophil infiltration time-dependently into BALF compared to the control. However, PLAG/LPS co-treated animals more rapidly return to homeostasis, showing baseline numbers of neutrophils in BALF by 16 h post-treatment. The amount of neutrophils transmigrated to the BALF at 16 h after LPS intranasal injection decreased in a dose-dependent manner in pretreated PLAG (10, 50, 250 mg/kg) (**Figure 1F**). PLAG treatment alone has no effect on neutrophil migration, and PLAG/LPS co-treatment does not alter neutrophil release from bone marrow or apoptosis (**Supplementary Figures 1b–e**). Thus, these data indicate that PLAG can specifically modulate excessive neutrophil infiltration into the lung.

To more precisely determine the role of PLAG in controlling excessive neutrophil infiltration into lung tissue in our ALI model, we measured the expression of several inflammation-related molecules in BALF cells and lung-homogenized tissue after treatment with PLAG and/or LPS for 16 h. We found that mRNA expression levels of MIP-2 [CXC Chemokine Ligand 2 (CXCL2)], a main factor involved in neutrophil migration, as well as S100A8 and S100A9, are increased in BALF cells from mice treated with LPS for 16 h compared to those from control animals (**Figures 1G–I** and **Supplementary Figure 2c**). This increased gene expression, however, is significantly attenuated in mice co-treated with PLAG/LPS for 16 h. Additionally several chemokines such as CXCL5, C-C motif chemokine ligand 2 (CCL2), or CCL5 exhibit a significant decrease of mRNA levels following treatment of PLAG (**Supplementary Figures 2b,c**). However, the reduction of CXCL5 and CCL2 mRNA expression after LPS/PLAG co-treatment was a small difference compared with the difference of the decrease expression levels of MIP-2 gene in BALF cells. The mRNA expression levels of CCL5, a key chemokine involved monocytes/macrophages migration is increased in BALF cells by LPS treatment and significantly decreased in mice co-treated with PLAG/LPS for 16 h. Other chemokines and their receptors exhibit either a moderate decrease or no change following treatment with PLAG (**Supplementary Figures 2a,b**). Further, we found that secreted levels of MIP-2 are also significantly increased in BALF following LPS administration, and markedly decreased by co-treatment with PLAG (**Figure 1I**). We have confirmed that inhibitors of CXC chemokine receptor 2 (CXCR2), a MIP-2 binding receptor, also completely reduced neutrophil infiltration to the lung of mice with LPS treatment (data not shown). The major inflammatory cytokine, IL-6 was also decreased in BALF and serum with PLAG administration (**Supplementary Figure 2d**). It is reported that LPS-induced TLR4 stimulation increases IL-6 expression (23). Thus, these results suggest that PLAG exerts an anti-inflammatory effect by blocking excessive neutrophil infiltration, at least in part, through the modulation of MIP-2 expression.

PLAG Induces More Rapid Endocytosis and Recovery of TLR4 Than LPS Alone and Promotes Clearance of Engulfed LPS

We next investigated the role of PLAG in ALI protection using an *in vitro* cell culture system. In RAW264.7 cells, a mouse macrophage cell line, LPS stimulates its cognate receptor TLR4 and subsequently induces LPS engulfment with aid of this receptor. Here, we evaluated the effect of PLAG on internalization of the LPS/TLR4 complex by analysis of surface spanning TLR4 using anti-TLR4/MD2 antibodies. PLAG-treated Raw264.7 cells show a more rapid endocytosis of the LPS/TLR4 complex and earlier recovery of TLR4 on surface membranes than those treated with LPS alone (**Figures 2A,B**). Specifically, initiation of TLR4 internalization can be clearly observed 30 min after treatment with LPS alone, whereas internalization is apparent after 15 min in cells co-treated with PLAG/LPS. Similarly, return of the TLR4 receptor to the cell surface membrane occurs 120 min after treatment with LPS and 60 min after co-treatment with PLAG/LPS. These data show that PLAG accelerates internalization of TLR4 receptor and promotes its return to the surface membrane.

We next investigated whether accelerated LPS/TLR4 endocytosis could lead to a more rapid clearance of intracellular LPS. It is well-known that, in macrophages, internalized LPS spontaneously stimulates generation of ROS, which function to eliminate the source of intracellular LPS. This also activates signaling pathways leading to production of numerous chemokines (mainly MIP-2) that recruit circulating neutrophils to the infection site. ROS generation is closely regulated by the NADPH oxidase system (24). Here, we found that in LPS-stimulated Raw264.7 cells, recruitment of p47phox, and Rac1 from the cytosol to the membrane can be observed at 30 min post-treatment and is sustained until 120 min post-treatment. In PLAG/LPS co-treated cells, the recruitment and return to homeostasis of both p47phox and Rac1 are observed at 15 and 60 min, respectively (**Figures 2C,D**). Similarly, ROS production can be detected 15 min earlier in PLAG/LPS-treated cells compared to those treated with LPS alone, and return to homeostatic levels of intracellular ROS occurs at 60 and 120 min post-treatment, respectively, for the PLAG/LPS- and LPS-treated groups (**Figure 2E**). These results were further confirmed using a lysosomal activity detection kit (**Figure 2F**). Thus, our data reveal that PLAG accelerates endocytosis of LPS/TLR4 and promotes a more rapid recruitment of p47phox and Rac1 enzymes and ROS production. In addition, return to homeostasis occurs more rapidly PLAG co-treated cells. To determine the effect on LPS clearance, we measured levels of intracellular LPS and found both a more rapid uptake and a faster removal of LPS in PLAG-treated cells (**Figures 2G,H**). Our data therefore indicate that PLAG facilitates a more rapid LPS-induced TLR4 endocytosis and accelerates LPS-induced ROS production and return to homeostasis via earlier clearance of LPS from invading pathogen.

PLAG Works as a Vesicle and Advanced TLR4 Endocytosis by PLAG Was Dependent on LPL and GPI-HBP1

PLAG is acetylated DAG with palmitic and linoleic acid and spontaneously forms a vesicle under emulsification (**Figure 3A**). Vesicle is recognized by LPL secreted by peripheral tissue resident cells. LPL bound vesicle is trapped by GPI-HBP1. GPI-HBP1 is expressed in the most cell waiting for uptake of lipid molecules as an energy source. To verify PLAG has role as a vesicle in the rapid endocytosis and recovery of TLR4, Knockdown of LPL and GPI-HBP-1 in THP-1 cells was constructed by siRNA transfection. Gene silencing of LPL, GPI-HBP1, caveolin-1, and clathrin was confirmed by western blot analysis (**Figure 3A**). In the scramble RNA transfected cells as control, PLAG/LPS treatment enables to rapid endocytosis at 15 min and recovery of TLR4 at 60 min when compared to only LPS treatment (**Figure 3B**). In the LPL or GPI-HBP1 gene silenced cells, added PLAG effect on rapid endocytosis and recovery of TLR4 was not observed (**Figures 3C,D**). In the caveolin-1 or clathrin silenced

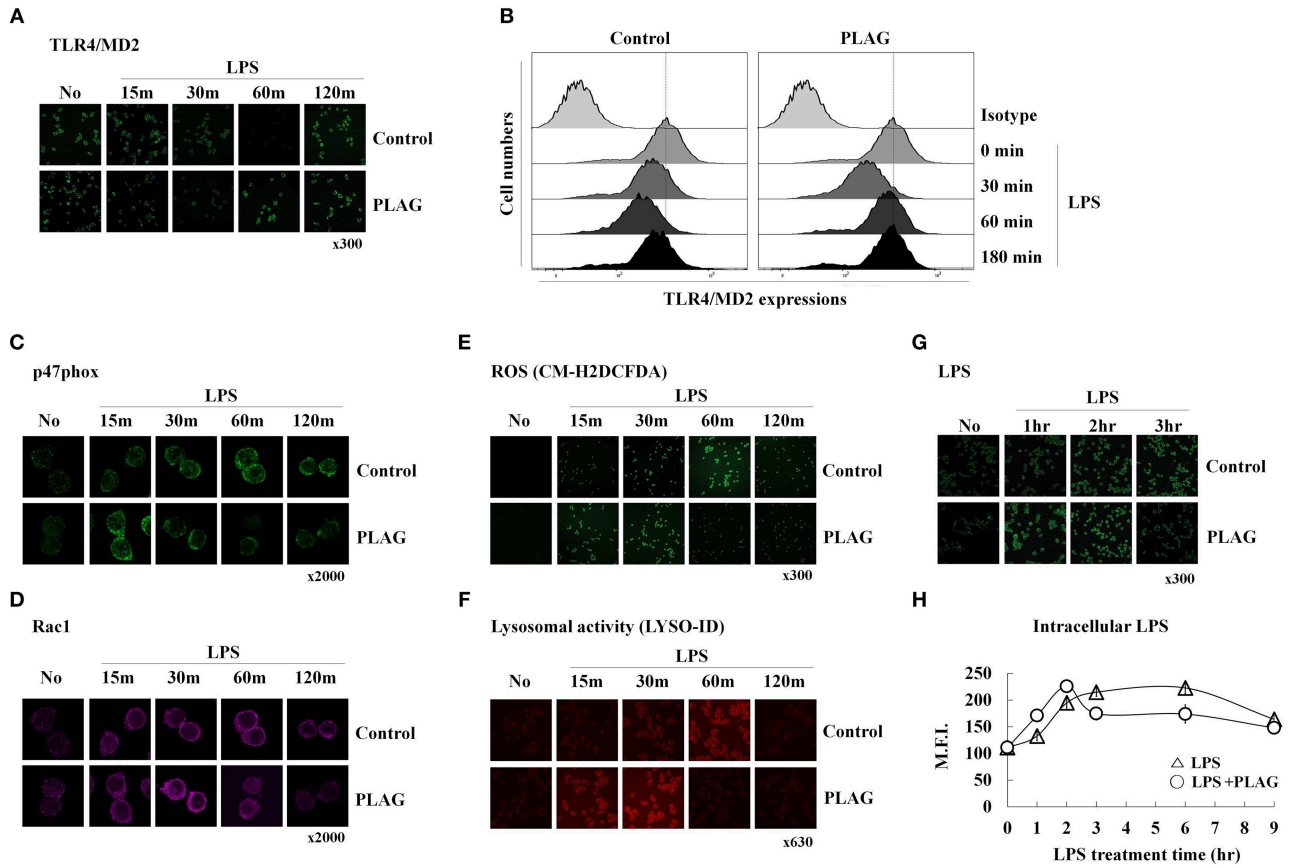


FIGURE 2 | PLAG accelerates LPS-induced TLR4 endocytosis and rapid LPS clearance. Raw264.7 cells were treated with 100 μg/ml of PLAG or DMSO (as solvent control) for 1 h and treated with 100 ng/ml LPS for 15, 30, 60, 120, and 180 min. Cells were then fixed and stained using rat anti-TLR4/MD2 antibody with Alexa488-conjugated anti-rat IgG secondary antibody. These were analyzed by confocal microscopy **(A)** and flow cytometry **(B)**. Raw264.7 cells stimulated under the same conditions were fixed, permeabilized, and stained with rabbit anti-p47phox **(C)** and mouse anti-Rac1 **(D)**, CM-H2DCFDA **(E)**, or the LYSO-ID Lysosomal Detection Kit **(F)**. Confocal microscopy was performed; all data shown represent one experiment performed in triplicate. Raw264.7 cells were treated with 100 μg/ml of PLAG or DMSO (as solvent control) for 1 h and stimulated with 100 ng/ml LPS for 1, 2, 3, 6, and 9 h **(G,H)**. Cells were then fixed, permeabilized, and stained with mouse anti-LPS antibody and Alexa488 conjugated anti-mouse IgG secondary antibody. These were analyzed by confocal microscopy and flow cytometry. Data represent one experiment performed in triplicate.

cells, endocytosis of TLR4 was not found (**Figures 3E,F**). These results suggest that PLAG is working as a form of micelles and has biological effects on the peripheral tissue containing cells which express LPL, GPI-HBP-1.

PLAG Affects TRIF-Dependent Endosomal Signaling Rather Than the Myd88 Pathway After LPS Stimulation

LPS binding to the TLR4/MD2 complex activates two distinct signaling pathways; the Myd88-dependent network is associated with membrane-localized TLR4 receptors, whereas the TRIF-dependent pathway is associated with endosomal signaling (**Figure 4A**) (25). Above, we observed that treatment with PLAG attenuates MIP-2 expression in lung tissue and BALF from LPS-treated mice, which is mainly dependent on the TRIF adaptor protein (**Figures 1G–I**). Therefore, to determine whether PLAG modulates the TRIF- and/or the MyD88 dependent pathways, Raw264.7 cells were transfected with specific siRNAs targeting Myd88 and TIRAP, to block Myd88-dependent signaling, and siRNAs for TRIF and TRAM, to inhibit TRIF-dependent signaling; target mRNAs were all effectively downregulated in siRNA-transfected cells (**Figure 4B**). Notably, in TRIF- and TRAM-silenced cells, the ability of PLAG to modulate production of MIP-2 and IFN-β is completely abolished (**Figure 4C**). However, in cells silenced for TIRAP or Myd88, the effects of PLAG effect could still be observed in a dose-dependent manner. This suggests that the ability of PLAG to modulate MIP-2 and IFN-β expression is mainly dependent on TRIF and the endosomal-associated signaling pathway. In contrast, LPS-induced expression of IL-1β and TNF, which is mediated by membrane-localized TLR4 receptor, is not modulated by PLAG (**Figure 4C**).

Modulation of TRIF signaling by PLAG in LPS-treated cells was further investigated by assessing the modification of TLR4-associated adaptor molecules. Assembled TLR4 adaptor proteins

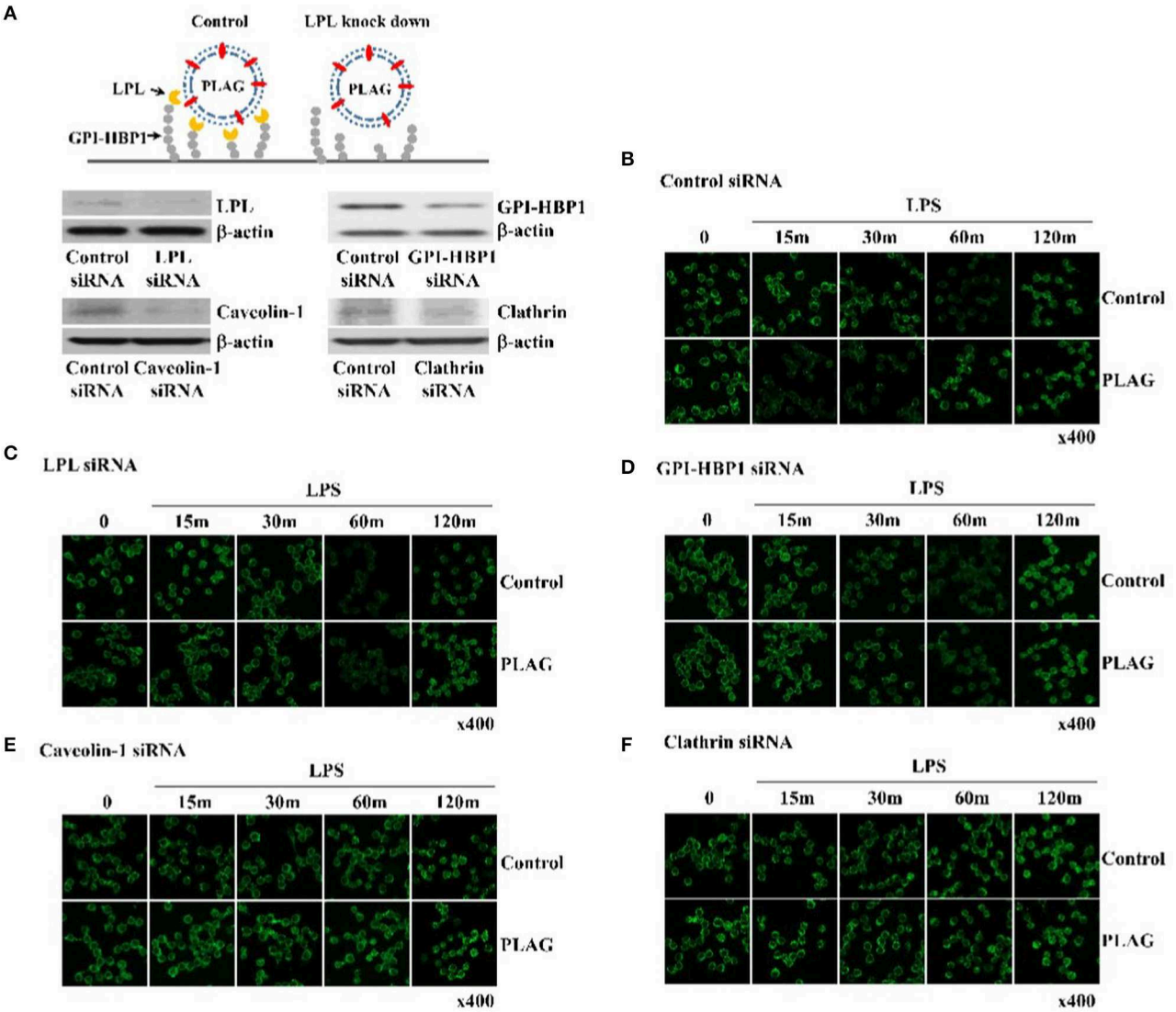


FIGURE 3 | PLAG has an effect on accelerated TLR4 endocytosis via LPL and GPI-HBP1 dependent pathway. Raw264.7 cells were transfected with LPL, GPI-HBP1, caveolin-1, or clathrin siRNA, respectively. After 24 h, LPL, GPI-HBP1, caveolin-1, or clathrin levels were assessed by western blot **(A)** and cells were incubated with 100 ug/ml of PLAG or DMSO (as solvent control) for 1 h. LPS was treated with 100 ng/ml for 15, 30, 60, 120 min, cells were fixed, permeabilized, and stained using rat anti-TLR4/MD2 antibody and Alexa488 conjugated anti-rat IgG as secondary antibody. Cells were analyzed confocal analysis **(B–F)**. β-actin was used as a control. All data shown represent one experiment performed in triplicate.

were precipitated using anti-TLR4/MD2 antibodies in LPS-stimulated cells with or without PLAG. Association of TRIF with TLR4/MD2 was detected at 30 min post-treatment and maintained up to 120 min in LPS-treated cells (**Figure 4D**). In PLAG/LPS co-stimulated cells, TRIF and TLR4 assembly initiates at 15 min post-treatment and complex disassembly was detected 60 min after LPS treatment. Conversely, association between Myd88 and TLR4 are unchanged in PLAG/LPS- vs. LPS-stimulated cells. These data demonstrate that PLAG accelerates the association and disassociation of TRIF and TLR4.

TRAM activation via phosphorylation recruits the TRIF adaptor molecule to the TLR4 endosomal-signaling pathway (26). Using anti-phospho-TRAM antibodies and western blot analysis, we detected TRAM phosphorylation at 30 min post-treatment, and this is sustained for up to 120 min in LPS-treated cells (**Figure 4E**). In PLAG/LPS-treated cells, phosphorylation of TRAM was found at 15 min post-treatment and terminates by 60 min. Notably, this earlier activation and faster resolution of TRAM phosphorylation in response to PLAG/LPS stimulation was found to be specific for this molecule, as the phosphorylation

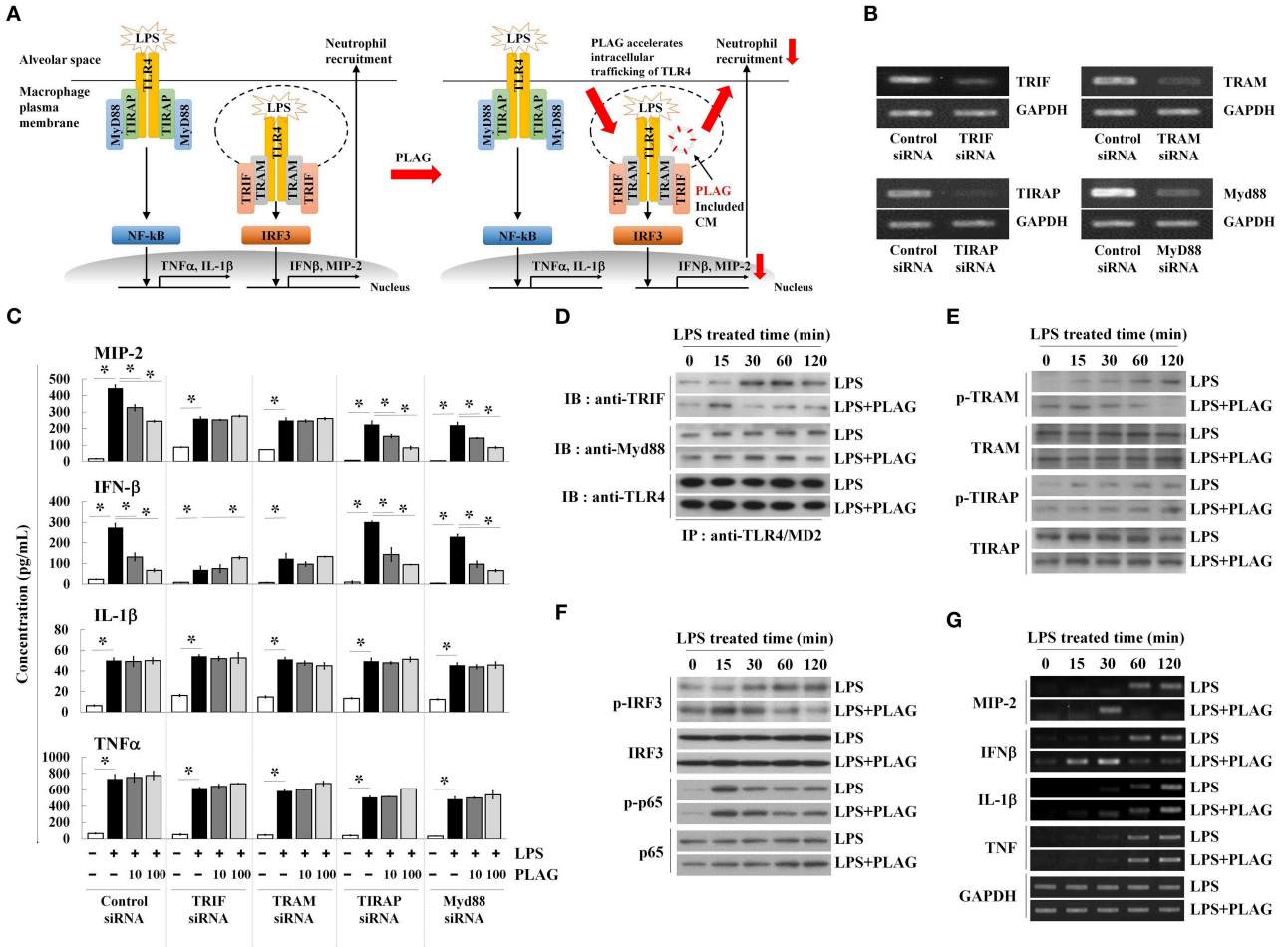


FIGURE 4 | PLAG accelerates the LPS-induced TRIF-dependent endocytosis pathway. Schematic diagram of TLR4-mediated signaling in response to LPS and PLAG/LPS in macrophages. TLR4 endocytosis by LPS treatment occurs and MIP-2 is produced by internalization signaling of TLR4, and neutrophils enter the alveoli. In the macrophages treated with PLAG, TLR4 intercellular trafficking by LPS is promoted and the internalization signaling of TLR4 is terminated rapidly, resulting in decreased expression of MIP-2, and reduction of neutrophil infiltration **(A)**. Raw264.7 cells were transfected with siRNA targeting TRIF, TRAM, TIRAP, and Myd88, as well as the scrambled control. After 24 h, target mRNA levels were assessed by RT-PCR **(B)**. Transfected cells were then incubated with 10 or 100 μg/ml of PLAG or DMSO (as solvent control) for 1 h and then stimulated with 100 ng/ml LPS for 16 h. Culture supernatants were assayed by ELISA to measure the secreted levels of MIP-2, IFN-β, IL-1β, and TNF **(C)**. Raw264.7 cells were incubated with 100 μg/ml of PLAG or DMSO (as solvent control) for 1 h and then stimulated with 100 ng/ml LPS for 15, 30, 60, and 120 min. Following immunoprecipitation with TLR4/MD2 antibodies, cell lysates were separated by SDS-PAGE and analyzed by immunoblot analysis with antibodies to TRIF, Myd88, and TLR4 **(D)**. The whole lysates were analyzed by immunoblot analysis to assess phosphorylation of TRAM or TIRAP **(E)** and IRF3 or p65 **(F)**. Total mRNA was extracted from whole cells, and expression of MIP-2, IFN-β, IL-1β, and TNF was assessed by RT-PCR **(G)**. GAPDH used as loading control. All Data represent one experiment performed in triplicate; *indicates $p < 0.05$.

of TIRAP is unchanged by addition of PLAG in LPS-stimulated cells (**Figure 4E**).

IRF3 and NF-κB are the main transcription factors activated in LPS-stimulated cells; these mediate activity of the endosomal TLR4 receptor-dependent signaling pathway and the membrane-localized TLR4 receptor-dependent signaling pathway, respectively. Phosphorylation of IRF3 in response to LPS stimulation can be detected at 30 min post-treatment and is maintained up to 120 min (**Figure 4F**). Co-treatment with PLAG, however, accelerates IRF3 phosphorylation, resulting in a peak at 15 min post-treatment, followed by a return to baseline 60 min after co-stimulation. Consistent with these data, MIP-2 and IFN-β mRNA transcripts, which are largely dependent on the TRIF-dependent TLR4 signaling pathway, were detected at 60 min post-stimulation and are sustained for 120 min in LPS-treated cells (**Figure 4G**). PLAG co-treatment accelerates the appearance of MIP-2 and IFN-β transcripts, and their levels return to control levels by 60 min. Consistent with our siRNA data, we find that PLAG preferentially modulates expression of MIP-2 and IFN-β, as the levels of IL-1β and TNF mRNA are unaffected by PLAG. Collectively, these data suggest that rather than acting as inhibitor, PLAG accelerates the response to LPS, specifically TLR4-mediated endosomal signaling, and returns cells to homeostasis in a shorter period of time.

PLAG Functions via Micelle Formation in an Endocytosis-Dependent Manner

PLAG is an acetylated DAG molecule that is utilized as a structural lipid during micelle formation. Similar to phosphatidylcholine, emulsified PLAG spontaneously forms micelles *in vitro* (data not shown). One type of micelle, known as a chylomicron, is generated in enterocytes and delivered to peripheral tissues where fatty acids in the chylomicron are distributed to cells with the aid of LPL and GPI-HBP1. Dietary PLAG is also absorbed by enterocytes and delivered as a component of chylomicrons into peripheral tissue (data not shown). Because LPS-induced MIP-2 is modulated by PLAG, we hypothesized that modulation of MIP-2 expression in macrophages by PLAG might be dependent on micelle formation. To test this, Raw264.7 cells were transfected with siRNA targeting LPL or GPI-HBP1, and the downregulation of their mRNA levels was confirmed in transfected cells (**Figure 5A**). We then found that the decreased secretion of MIP-2 and IFN-β observed PLAG/LPS-treated cells as compared to cells treated with LPS alone was not observed in cells silenced for LPL or GPI-HBP1 (**Figure 5B**); IL-1β and TNF are unaffected. These data suggest that PLAG may function in micelles, which are recognized by LPL and communicate with cells via GPI-HBP1.

We next transfected Raw264.7 cells with siRNAs targeting clathrin and caveolin-1, which are involved in TLR4-mediated endocytosis, and confirmed their downregulation in siRNA-transfected cells by RT-PCR (**Figure 5C**). As noted above, PLAG co-treatment differentially decreases secretion of MIP-2 and IFN-β in LPS-stimulated cells, but has no effect on IL1-β and TNF (**Figure 5D**). In clathrin and caveolin-1 silenced cells, however, secretion of MIP-2 and IFN-β is unaffected by PLAG treatment. These data confirm that the biological role of PLAG in the modulation of MIP-2 chemokine expression is dependent on micelle formation and internalization of the TLR4 receptor.

PLAG Blocks Neutrophil-Mediated Endothelial Permeability *in vitro*

The prominent phenotype of ALI is the massive infiltration of neutrophils into the bronchoalveolar tissues, which results in damage to the endothelial cells and/or loss of the endothelial barrier (27). We therefore investigated whether PLAG protects the integrity of the endothelial barrier using a Transwell assay. For neutrophil cells, we used differentiated HL-60 cells, which were cultured in the upper Transwell chambers. HUVEC cell were plated on the Transwell inserts to act as endothelial barrier cells, and THP-1 cells were used as chemokine-releasing cells. Culture supernatants from LPS-stimulated THP-1 cells contain secreted chemokines, particularly IL-8, and these were placed in bottom Transwell chamber (**Figure 6A**). In this assay, HL-60 cells in the upper chamber move toward the lower chamber along chemokine gradients, which are provided by the supernatant from the LPS-stimulated THP-1 cells. If neutrophil transmigration occurs, proteins in the upper chamber move across the endothelial HUVEC layer to lower chamber. Loading of streptavidin-HRP in the upper chamber stains the plasma proteins in cell supernatant, similar to Evans blue dye staining of albumin *in vivo*. The migrated plasma proteins in the lower chamber can then be detected by TMB substrate, and this can be used to assess endothelial permeability. Using this assay, we found that supernatant from PLAG/LPS-treated THP-1 cells promote a markedly decreased level of LPS-induced endothelial permeability and dHL-60 cell migration, with PLAG acting in a dose-dependent manner (**Figures 6B–D**). These results were confirmed by measuring migration of mouse bone marrow-derived primary neutrophils in the same assay (**Figures 6E,F**). Inhibitors of TLR4 or NOX were further shown to inhibit primary neutrophil transmigration (**Supplementary Figure 3**). Thus, our data indicate that PLAG can block excessive neutrophil migration into bronchoalveolar tissues.

Confirmation of PLAG Therapeutic Specificity by Use of PLAG Metabolites

To determine specificity of PLAG, an acetylated DAG, we assessed the therapeutic efficacy of PLAG metabolites and compared their biological efficacy in our animal model *in vivo*. 1-palmitoyl-2-linoleoyl-3-hydroxyl-rac-glycerol (PLH) is a DAG that consists of two fatty acid chains, palmitic acid and linoleic acid. 1-hydroxyl-2-linoleoyl-3-hydroxyl-glycerol (HLH) is composed of linoleic acid and a glycerol backbone. Linoleic acid (LA) or palmitic acid (PA) was also used (**Figure 7A**). In our ALI animal model, LPS treatment via intranasal administration induces massive neutrophil extravasation into the alveolar cavity, which is easily detected in the BALF. PLAG co-treated mice show a dramatically reduced number of neutrophils in the BALF, and counts rapidly return to a normal status (**Figures 1C**, **7B**). Conversely, PLH, HLH, LA, and PA have no effect on the number of neutrophils in BALF from LPS-treated mice (**Figure 7B**). These data indicate that PLAG has a specific role in blocking the excessive and sustained neutrophil infiltration during LPS-induced ALI progression. In LPS-stimulated Raw264.7 cells, TLR4/MD2 internalization is observed at 30 min post-treatment and sustained for up to 120 min. In contrast, PLAG co-treated cells show increased TLR4/MD2 internalization at 15 min, and TLR4 returns to the surface at 60 min. However, co-treatment with PLH has no effect on TLR4/MD2 internalization and return to the surface (**Figure 7C**). These findings suggest that the acetylation of DAG is a critical factor in blocking excessive neutrophil infiltration in the ALI animal model and.

PLAG exerts a therapeutic effect in the ALI mouse model when co-administered with LPS. Therefore, we next investigated whether PLAG has the same effect when administered post-treatment. To test this, mice were treated with LPS for 2 h, then PLAG was then administrated orally (**Figure 7D**). We found that the number of infiltrated neutrophils in BALF was diminished by half in the post-treated mice, as compared to those treated with LPS alone (**Figure 7E**). MIP-2 levels were also significantly decreased in BALF from post-treated group (**Figure 7F**). Thus, we propose that PLAG may hold potential a therapeutic agent for treatment of inflammatory diseases, such as ALI.

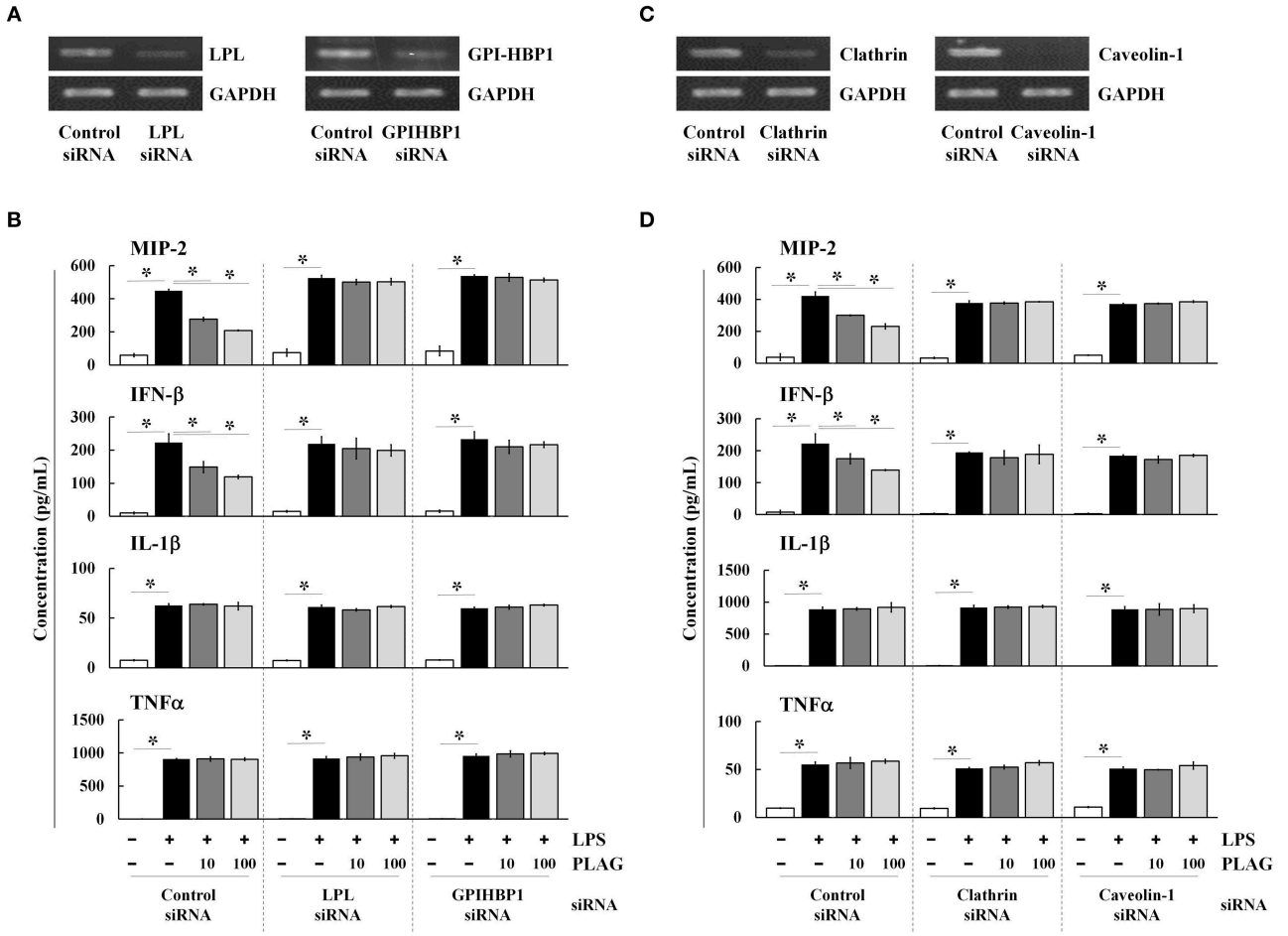


FIGURE 5 | PLAG functions via micelle formation. Raw264.7 cells were transfected with siRNA targeting LPL or GPI-HBP1, and after 24 h, expression of target genes was assessed by RT-PCR **(A)**. Transfected cells were incubated with 10 or 100 μg/ml of PLAG or DMSO (as solvent control) for 1 h and then treated with 100 ng/ml LPS for 16 h. Culture supernatants were assayed by ELISA to measure secreted levels of MIP-2, IFN-β, IL-1β, and TNF **(B)**. Raw264.7 cells were transfected with siRNA targeting clathrin or caveolin-1, and after 24 h, expression of target genes was assessed by RT-PCR **(C)**. Transfected cells were then treated with 10 or 100 μg/ml of PLAG or DMSO (as solvent control) for 1 h and stimulated with 100 ng/ml LPS for 16 h. Culture supernatants were analyzed by ELISA assay to measure secreted levels of MIP-2, IFN-β, IL-1β, and TNF **(D)**. Data represent one experiment performed in triplicate. *indicates $p < 0.05$.

DISCUSSION

In this study, we tested the therapeutic efficacy of the acetylated DAG, PLAG, for preventing disease pathology in a mouse model of ALI and observed significantly reduced lung inflammation in animals co-treated with PLAG/LPS, as compared to LPS alone (**Figure 1**). Histological examination showed that severe pulmonary destruction, including bronchial alveolar tissue damage and massive leukocyte infiltration, were observed in the LPS-treated mice. In PLAG/LPS-treated mice, however, LPS-induced ALI was not observed, and the morphology of all lung tissue was similar to that of control mice. In addition, post-treatment with PLAG also reduces the number of infiltrating neutrophils in the alveolar tissue in this ALI mouse model (**Figures 7D–F**). Further, we found that PLAG, an acetylated DAG that is also found in nature, has a unique biological effect in reducing the amount of infiltrating neutrophils into the alveolar tissue when compared with a natural DAG (PLH), and other PLAG metabolites (**Figure 7B**). These findings suggest that PLAG might hold potential as a therapeutic agent for prevention of lung inflammation.

PLAG is a synthetic monoacetyldiacylglyceride (17) and a small lipid molecule. In our reports, the concentrations of PLAG in plasma, whole blood, or/and lymph of rat, dog and monkey were obtained measured using the LS-MS/MS method following oral administration of a range of 500 between 4,000 mg/kg of PLAG (unpublished data). Lymphatic exposure of PLAG after oral administration is 10–100-fold increase than blood exposure, and the biggest difference in the PLAG exposure between lymph and blood system was observed in monkeys. Generally, lipid is absorbed through enterocytes, reconstructed with chylomicron, delivered to lymph, and then exposed to blood system, and distributed throughout the body. To sum up the above mentioned results, more than 75% of dosed PLAG

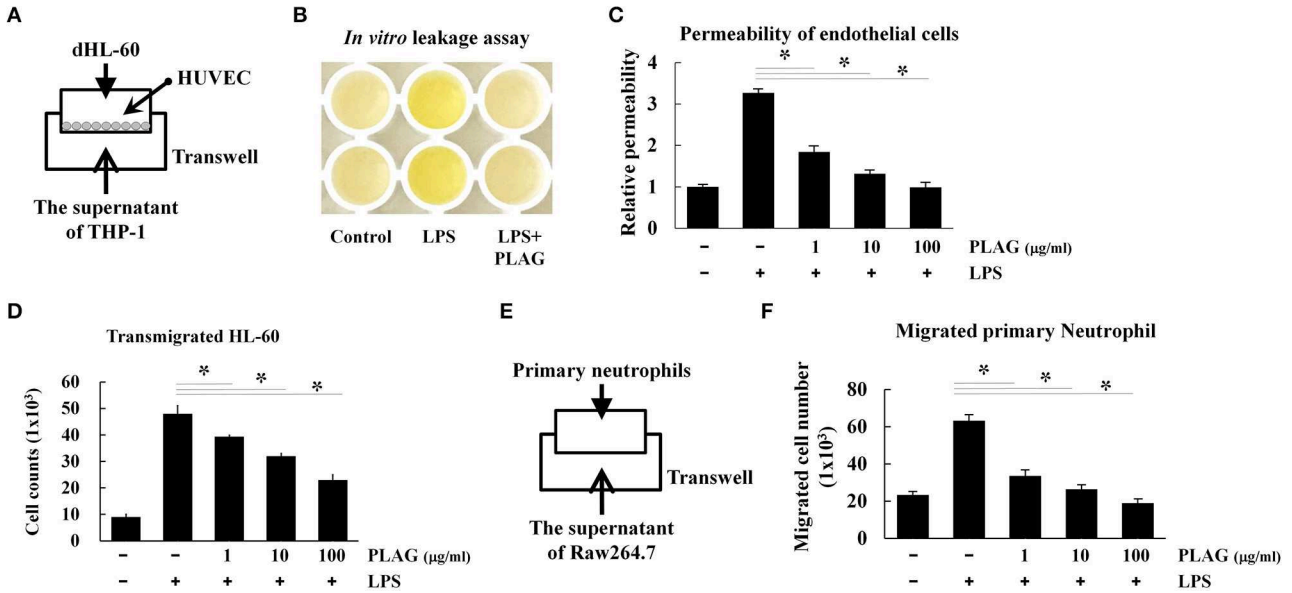


FIGURE 6 | PLAG regulates the excessive vascular leakage of endothelial permeability and neutrophil migration. HUVEC cells (1×10^4) were seeded onto 5-μm pore Transwell inserts, and after 16 h, the supernatant was removed and replaced with media containing differentiated HL-60 cells **(A)**. The lower Transwell chamber was loaded with the supernatant of THP-1 cells that had been pre-incubated with PLAG (100 μg/ml) for 1 h and then stimulated with LPS (100 ng/ml) for 16 h. The combined Transwells were incubated at 37°C for 6 h, and then *in vivo* leakage and endothelial cell permeability were analyzed by the Transwell permeability assay, as described in the Materials and Methods **(B,C)**. Migrated dHL-60 cells were also counted **(D)**. Primary neutrophils were isolated from bone marrow, and their transmigration ability was assessed using supernatant from Raw264.7 cells that were pre-incubated with PLAG (100 μg/ml) for 1 h and then with LPS (100 ng/ml) for 16 h **(E)**. Migrated primary neutrophils were counted **(F)**. All Data represent one experiment performed in triplicate; *indicates $p < 0.05$.

was excreted by 24 h and the Cmax 100 mg/kg was 38.8 μg eq./mL in blood and 1,210 μg eq./mL in lymph, respectively. The t1/2 100 mg/kg was 16.8 and 29.7 h, respectively. Based on these results, considering the time when PLAG is sufficiently exposed to the peripheral tissue, it was set at 16 h postdose to see the effects of removing LPS and then resolving systemic inflammation to improve ALI. We have already described in **Figure 1E** that the basis for selecting the ameliorating time of LPS-induced ALI to reduce neutrophil infiltration to the lung tissue was 16 h after the administration of PLAG. And we have shown that the most effective dose of PLAG in the improvement of ALI was 250 mg/kg. Based on our tests, a simple calculation shows that the concentration of PLAG in the blood of mice administered with 250 mg/kg PLAG is likely to be greater than about 100 μg/mL. Thus, for i*n vitro* assay, the biological effects were verified by treating cells directly at concentrations of 10 to 100 μg/mL PLAG.

Because PLAG is fat, the absorbed PLAG in the body is likely to decompose in the intestine and form metabolites through the recombination process. Therefore, we have checked the metabolite profiling of plasma and lymph fluid. Several peaks in the plasma and the lymph fluid were evaluated quantitatively and qualitatively. In the plasma, the predominant component was suggested to be glucose in the mass spectrometric analysis. In the lymph fluid, the predominant components were suggested to be a mixture of various triglyceride in the mass spectrometric analysis. Unchanged EC-18 and PLH (hydrolyzed form of acetate ester in EC-18) were also detected in the plasma and lymph fluid as minor components. However, we already have checked the effects of the metabolites (e.g., PLH, PA, LA, and HLH) on the neutrophil infiltration to the peripheral lung tissue in LPS-induced ALI model (**Figure 7B**). The metabolites of PLAG had no effect on the improvement of LPS-induced acute lung inflammation. In addition, the predominant components of the metabolite were suggested to be a mixture of various triglyceride. We have checked the effect of olive oil (composed with 13% saturated fats and >85% unsaturated fats) on the neutrophil infiltration to the inflamed peripheral tissue by the chemical damage (data not shown). Likewise, the administration of olive oil was not affected to improve the tissue inflammation. Taken together, we are convinced that the metabolites have little effect on the improving effects of LPS-induced ALI and that PLAG is the critical effect molecule.

MIP-2 is a known intermediary chemoattractant for neutrophils and plays a key role in inflammation-related diseases, including arthritis, cancers, and pulmonary disease (28–31). LPS stimulation of bronchoalveolar tissue induces secretion of numerous pro-inflammatory molecules, including MIP-2. This chemokine likely contributes to the initiation and extension of inflammatory process and is often abundantly detected at sites of tissue inflammation. Induction of MIP-2 by LPS treatment is mainly controlled by the TLR4-mediated endosome-dependent signaling pathway. Here, we found that PLAG exerts its biological effect by accelerating the endocytosis and exocytosis of TLR4. PLAG co-treatment promotes earlier

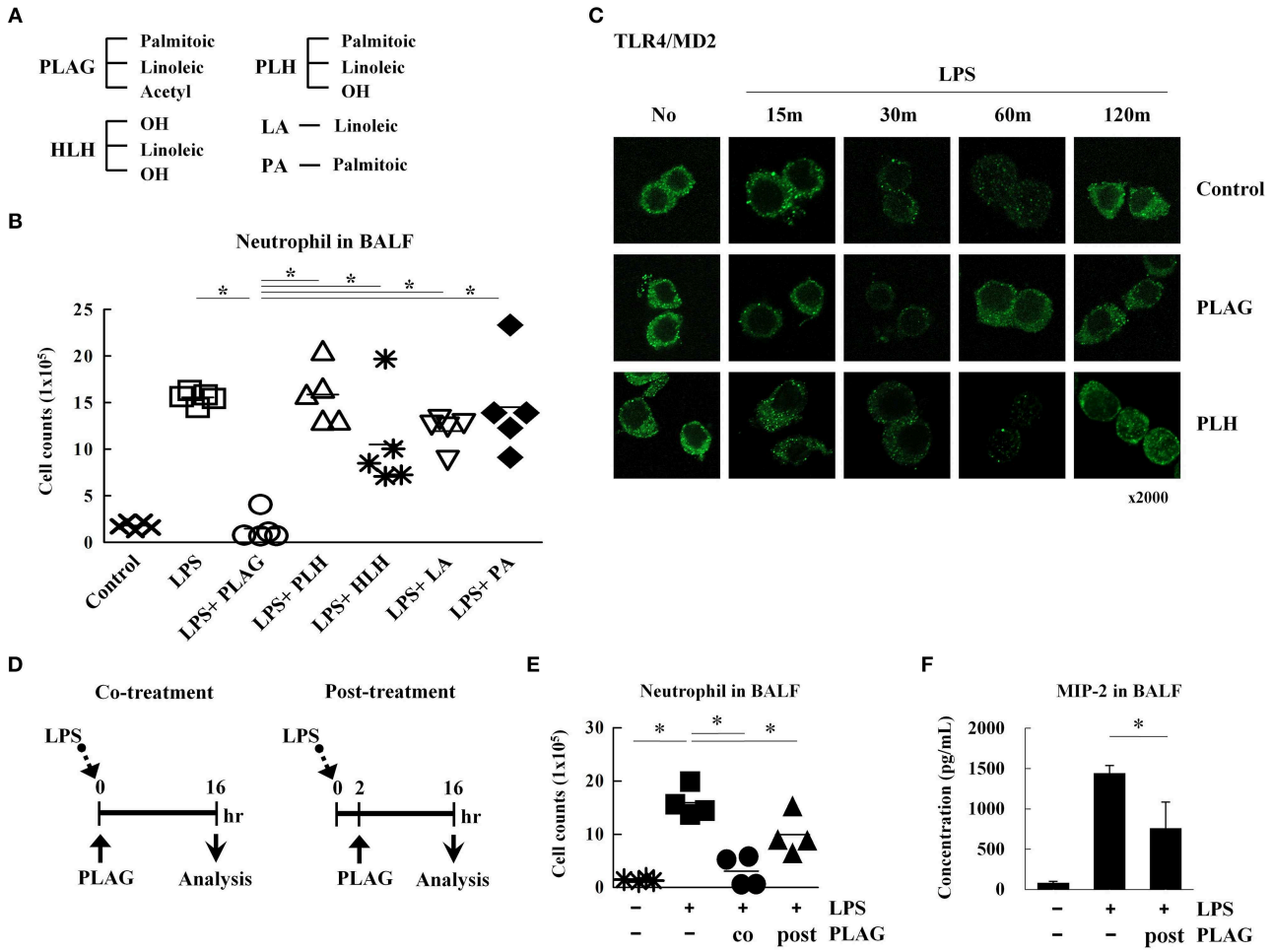


FIGURE 7 | The acetylation of diacylglycerol is critical for attenuating excessive neutrophil infiltration in the ALI model. Chemical structure diagram of PLAG and PLAG metabolites: PLH, HLH, LA, and PA **(A)**. Mice were divided into seven separate groups ($n = 5$ per group): control, LPS-treated, PLAG/LPS co-treated, LPS/PLH co-treated, LPS/HLH co-treated, LPS/LA co-treated, and LPS/PA co-treated. Mice were treated as described in **Figure 1A**. LPS (25 mg/kg) was administered by an intranasal route, and PLAG (250 mg/kg) or PLAG derivatives (250 mg/kg) were administered orally. After 16 h, BALF cells were counted **(B)**. Raw264.7 cells were incubated with 100 μg/ml PLAG, PLH, or DMSO (as solvent control) for 1 h and then treated with 100 ng/ml LPS for 15, 30, 60, or 120 min. Cells were fixed and stained with rat anti-TLR4/MD2 antibody and Alexa488-conjugated anti-rat IgG secondary antibody **(C)**. Mice were divided into four separate groups: control, LPS-treated, PLAG/LPS co-treated, and PLAG/LPS post-treated ($n = 4$ per group). LPS (25 mg/kg) was administered by an intranasal route, and PLAG (250 mg/kg) was administered orally. In the PLAG/LPS post-treated group, PLAG was administrated after 2 h of LPS treatment **(D)**. Approximately 16 h after LPS administration, mice were sacrificed, and the number of neutrophils in BALF were counted **(E)**. Levels of secreted MIP-2 in BALF were measured using an ELISA assay **(F)**. Data represent four mice samples from each group. *In vivo* experiments were from at least three independent experiments, and the bar represents the mean. *indicates $p < 0.05$.

expression of MIP-2 and endosome-related signals in LPS-stimulated Raw264.7 cells. Moreover, PLAG also induces a more rapid return of the TLR4 receptor to the plasma membrane. This suggests that PLAG effectively terminates endosome-dependent signaling and turns off MIP-2 expression in a shorter amount of time than in cells stimulated with LPS alone. Also, we confirmed that the use of inhibitors of CXCR2, a MIP-2 binding receptor, completely reduced neutrophil infiltration to the lung of mice with LPS treatment (data not shown). In this study, we focused that MIP-2 (CXCL2 or CXCL8) is a main chemoattractant for neutrophils. However, we also have confirmed that other LPS-induced chemokines such as CXCL5, CCL2, and CCL5 were reduced the mRNA expressions in BALF by PLAG co-treatment (**Supplementary Figure 2**). The reason is that LPS-induced ALI was promptly terminated by PLAG treatment. In other words, rapid resolution of acute inflammation by PLAG showed the reductions of other chemokines, its receptors, inflammatory cytokine and damage-associated molecular patterns expression in the lung of LPS-induced ALI mice. Generally, CCL2 and CCL5 are key chemokines that monocytes/macrophages to the site of inflammation (32). CXCL5 is well-known to chemotactic and activating functions on neutrophil and interacts with CXCR2 (33). CXCL5 is also known as epithelial-derived neutrophil activating peptide. Although we have checked only the mRNA

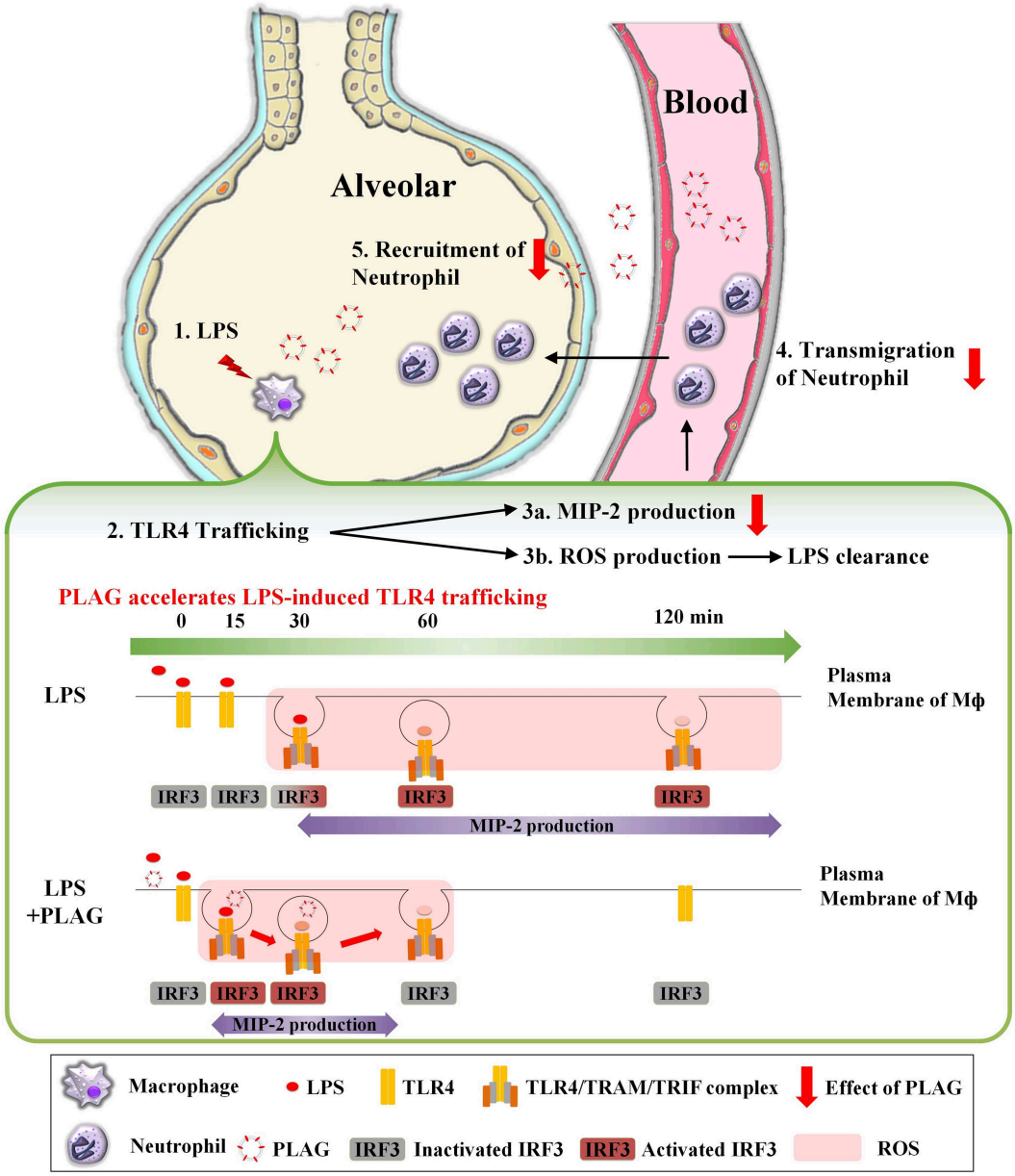


FIGURE 8 | PLAG prevents ALI through reduction of neutrophil transmigration via acceleration of TLR4 trafficking. A serial cascade is expected to occur following LPS treatment, leading to the recruitment of neutrophils into the alveolar. (1) Exogenously injected LPS binds to MD2/TLR4 on the surface of macrophage in the alveoli, resulting in activation of macrophage. (2) Activated TLR4 binds to endocytosis-dependent adaptor protein such as TRAM and TRIF, and internalized into intracellular. Then, IRF3, which is a downstream of TLR4/TRAM/TRIF complex, is activated and acts as a transcription factor in the nucleus, (3a) expressing mRNA of MIP-2 and induces the production of MIP-2 protein. MIP-2 produced from macrophage forms a concentration gradient to recruit neutrophils into alveoli. (3b) Internalization of TLR4 also produces intracellular ROS. The generated ROS remove LPS and MIP-2 induces (4) transmigration and (5) recruitment of neutrophils into alveolar. Finally, the superfluous recruited neutrophils will damage to host tissue and occur the inflammation. At this time, PLAG treatment promotes trafficking of TLR4 that occurs in response to LPS on plasma membrane of macrophage. Therefore, PLAG induces rapid removal of LPS by promoting ROS generation time and induced rapid removal of ROS. In addition, the duration of activation of IRF3 by internalization of TLR4 is reduced, which ultimately reduces the total amount of MIP-2 expression and prevents the excessive recruitment of neutrophils to the alveolar space. Upon PLAG administration, LPS-induced neutrophil transmigration is attenuated via acceleration of TLR4 trafficking.

expression of CXCL5 in BALF, it is possible that the decrease of CXCL5 expression also is regulated by PLAG. However, the reduction of CXCL5 mRNA expression after LPS/PLAG co-treatment was a small difference. Therefore, these data suggest that PLAG acts as pro-resolving molecule by limiting an excessive inflammatory response via effective control of MIP-2 expression. This is consistent with our previous reports, which showed PLAG efficacy in the control of inflammation. For example, PLAG improved survival in a mouse sepsis model, attenuated the infiltration of inflammatory cells into the airway

in a mouse asthma model, and exerted an anti-tumor effect in a hamster biliary cancer model (34–36).

LPS stimulates TLR4-mediated endocytosis, and as noted above, PLAG induces an earlier internalization of TLR4 in cells treated with LPS. This internalization of TLR4 is also critical for induction of MIP-2 expression. Evidence for the role of PLAG in promoting endocytosis of TLR4 was further obtained by demonstrating the earlier recruitment of NOX subunits, p47phox and Rac1, as well as earlier ROS generation in cells co-treated with PLAG/LPS compared to LPS alone. Notably, we further found that PLAG only exerts an effect on the TLR4 TRIF-dependent endosomal-signaling pathway and not on the Myd88-dependent pathway (**Figure 4**). The rapid induction and fast termination of TRIF-dependent TLR4 signaling by PLAG suggests that this molecule can promote a faster return to homeostasis, thus avoiding unnecessary and harmful inflammatory responses. We propose that this may promote optimal ROS production for clearance of invading pathogens without excessive host cell damage.

In this study, we developed the ALI mouse model by directly and intranasally injecting 25 mg/kg of LPS into the mice. LPS is a typical endotoxin derived from gram negative bacteria, and have been widely utilized for nearly 100 years in bioresearch area because it replicates much of physiology of infection-associated disease including ALI, ARDS, and sepsis (37, 38). Despite its simplicity in learning the molecular mechanism, we admit the fact that the use of LPS has several inherent limitations. First, LPS is one of components of various pathogen associated molecular patterns (PAMPs) produced by gram-negative microorganisms (37, 38). Therefore, LPS models neglects host interaction to other gram-negative-derived PAMPs and infection to gram positive bacteria and poly-microbes (37, 38). Second, ALI/ALDS models can be developed via non-infectious methods such as acid aspiration, hyperoxia, surfactant depletion and lung ischemia reperfusion (39). In order to overcome these limitations, we investigated whether PLAG shows the therapeutic efficacy in acute pneumonia models by challenging both gram-negative and -positive bacteria, and PLAG effectively regulated neutrophil infiltration and clearance of invaded pathogens (data not shown). In previous study, PLAG also effectively suppressed cigarette smoke-induced neutrophilic inflammation in lung tissue by regulating pro-inflammatory cytokine production and ROS generation (40). Based on these observations, we are confident that PLAG would have a similar therapeutic efficacy in multiple and different ALI animal models.

In summary, as shown in **Figure 8**, TLR4 on the surface of macrophage recognizes LPS injected into the alveolar space and internalized into cytosol. Internalization of TLR4 produces two responses. First, it induces TLR4/TRAM/TRIF/IRF3/MIP-2 signal cascade, an endocytosis dependent signaling pathway of TLR4. Second, ROS is generated to remove LPS. At this time, PLAG promotes LPS-induced TLR4 trafficking of macrophage surface. Therefore, by shortening the period of internalization of TLR4, it is possible to reduce the production of MIP-2, which acts as chemotaxis of neutrophils, to prevent excessive recruitment of neutrophils and to produce ROS in a shorter time, thereby promoting the clearance of LPS. Collectively, our data show that PLAG stimulates a more rapid resolution of LPS-induced lung inflammation, which suggests that it may hold potential as a therapeutic agent for various inflammatory diseases.

ETHICS STATEMENT

All animal experimental procedures were performed in accordance with the Guide and Use of Laboratory Animals (Institute of Laboratory Animal Resources). All experiments were approved by the Institutional Review Committee for Animal Care and Use of KRIBB (Korea Research Institute of Bioscience and Biotechnology, Daejeon, Republic of Korea), approval number KRIBB-AEC-16031.

AUTHOR CONTRIBUTIONS

JWK and H-RL: conception and design. H-RL, S-HS, and JHK: development of methodology. H-RL, S-HS, and JHK: acquisition of data. JWK, H-RL, S-HS, JHK, and SY: analysis and interpretation of data. K-YS: supporting and provision of materials. JWK, H-RL, and SY: writing, review, and/or revision of the manuscript.

FUNDING

This work was supported by the Korea Research Institute of Bioscience and Biotechnology (KRIBB) Research Initiative Program (KGM5251911), the ICORE project of University of Science and Technology (UST) (KFM0551711 and KFM0671811), and a grant (IGM0201811 and IGM 0171911) from ENZYCHEM Lifesciences.

ACKNOWLEDGMENTS

We thank Jinseon Jeong for her revision of the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.02177/full#supplementary-material

REFERENCES

1. Bernard GR, Artigas A, Brigham KL, Carlet J, Falke K, Hudson L, et al. The American-European Consensus Conference on ARDS. Definitions, mechanisms, relevant outcomes, and clinical trial coordination. *Am J Respir Crit Care Med.* (1994) 149:818–24. doi: 10.1164/ajrccm.149.3.7509706
2. Bian Z, Guo Y, Ha B, Zen K, Liu Y. Regulation of the inflammatory response: enhancing neutrophil infiltration under chronic inflammatory conditions. *J Immunol.* (2012) 188:844–53. doi: 10.4049/jimmunol.1101736
3. Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, et al. Neutrophil extracellular traps kill bacteria. *Science.* (2004) 303:1532–5. doi: 10.1126/science.1092385

4. Urban CF, Ermert D, Schmid M, Abu-Abed U, Goosmann C, Nacken W, et al. Neutrophil extracellular traps contain calprotectin, a cytosolic protein complex involved in host defense against Candida albicans. *PLoS Pathog.* (2009) 5:e1000639. doi: 10.1371/journal.ppat.1000639
5. Hilda JN, Das SD. TLR stimulation of human neutrophils lead to increased release of MCP-1, MIP-1alpha, IL-1beta, IL-8 and TNF during tuberculosis. *Hum Immunol.* (2015) 77:63–7. doi: 10.1016/j.humimm.2015.10.005
6. Kruger P, Saffarzadeh M, Weber AN, Rieber N, Radsak M, Von Bernuth H, et al. Neutrophils: Between host defence, immune modulation, and tissue injury. *PLoS Pathog.* (2015) 11:e1004651. doi: 10.1371/journal.ppat.1004651
7. Thomas L. The physiological disturbances produced by endotoxins. *Annu Rev Physiol.* (1954) 16:467–90. doi: 10.1146/annurev.ph.16.030154.002343
8. Beutler B, Rietschel ET. Innate immune sensing and its roots: the story of endotoxin. *Nat Rev Immunol.* (2003) 3:169–76. doi: 10.1038/nri1004
9. Bedard K, Krause KH. The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. *Physiol Rev.* (2007) 87:245–313. doi: 10.1152/physrev.00044.2005
10. Leverence JT, Medhora M, Konduri GG, Sampath V. Lipopolysaccharide-induced cytokine expression in alveolar epithelial cells: role of PKCzeta-mediated p47phox phosphorylation. *Chem Biol Interact.* (2011) 189:72–81. doi: 10.1016/j.cbi.2010.09.026
11. Kobayashi Y. The role of chemokines in neutrophil biology. *Front Biosci.* (2008) 13:2400–7. doi: 10.2741/2853
12. Wengner AM, Pitchford SC, Furze RC, Rankin SM. The coordinated action of G-CSF and ELR + CXC chemokines in neutrophil mobilization during acute inflammation. *Blood.* (2008) 111:42–9. doi: 10.1182/blood-2007-07-099648
13. Akira S, Takeda K. Toll-like receptor signalling. *Nat Rev Immunol.* (2004) 4:499–511. doi: 10.1038/nri1391
14. Horng T, Barton GM, Flavell RA, Medzhitov R. The adaptor molecule TIRAP provides signalling specificity for Toll-like receptors. *Nature.* (2002) 420:329–33. doi: 10.1038/nature01180
15. Fitzgerald KA, Rowe DC, Barnes BJ, Caffrey DR, Visintin A, Latz E, et al. LPS-TLR4 signaling to IRF-3/7 and NF-kappaB involves the toll adapters TRAM and TRIF. *J Exp Med.* (2003) 198:1043–55. doi: 10.1084/jem.20031023
16. Yang HO, Kim SH, Cho SH, Kim MG, Seo JY, Park JS, et al. Purification and structural determination of hematopoietic stem cell-stimulating monoacetyldiglycerides from Cervus nippon (deer antler). *Chem Pharm Bull.* (2004) 52:874–8. doi: 10.1248/cpb.52.874
17. Yang HO, Park JS, Cho SH, Yoon JY, Kim MG, Jhon GJ, et al. Stimulatory effects of monoacetyldiglycerides on hematopoiesis. *Biol Pharm Bull.* (2004) 27:1121–5. doi: 10.1248/bpb.27.1121
18. Yoo N, Lee HR, Shin SH, Sohn KY, Kim HJ, Han YH, et al. PLAG (1-palmitoyl-2-linoleoyl-3-acetyl-rac-glycerol) augments the therapeutic effect of pegfilgrastim on gemcitabine-induced neutropenia. *Cancer Lett.* (2016) 377:25–31. doi: 10.1016/j.canlet.2016.04.025
19. Lee HR, Yoo N, Kim JH, Sohn KY, Kim HJ, Kim MH, et al. The therapeutic effect of PLAG against oral mucositis in hamster and mouse model. *Front Oncol.* (2016) 6:209. doi: 10.3389/fonc.2016.00209
20. Matute-Bello G, Downey G, Moore BB, Groshong SD, Matthay MA, Slutsky AS, et al. An official American Thoracic Society workshop report: features and measurements of experimental acute lung injury in animals. *Am J Respir Cell Mol Biol.* (2011) 44:725–38. doi: 10.1165/rcmb.2009-0210ST
21. Saito T, Yamamoto T, Kazawa T, Gejyo H, Naito M. Expression of toll-like receptor 2 and 4 in lipopolysaccharide-induced lung injury in mouse. *Cell Tissue Res.* (2005) 321:75–88. doi: 10.1007/s00441-005-1113-9
22. Birukova AA, Wu T, Tian Y, Meliton A, Sarich N, Tian X, et al. Iloprost improves endothelial barrier function in lipopolysaccharide-induced lung injury. *Eur Respir J.* (2013) 41:165–76. doi: 10.1183/09031936.00148311
23. Wu TT, Chen TL, Chen RM. Lipopolysaccharide triggers macrophage activation of inflammatory cytokine expression, chemotaxis, phagocytosis, and oxidative ability via a toll-like receptor 4-dependent pathway: validated by RNA interference. *Toxicol Lett.* (2009) 191:195–202. doi: 10.1016/j.toxlet.2009.08.025
24. Segal BH, Leto TL, Gallin JI, Malech HL, Holland SM. Genetic, biochemical, and clinical features of chronic granulomatous disease. *Medicine.* (2000) 79:170–200. doi: 10.1097/00005792-200005000-00004
25. Yamamoto M, Sato S, Hemmi H, Hoshino K, Kaisho T, Sanjo H, et al. Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. *Science.* (2003) 301:640–3. doi: 10.1126/science.1087262
26. Oshiumi H, Sasai M, Shida K, Fujita T, Matsumoto M, Seya T. TIR-containing adapter molecule (TICAM)-2, a bridging adapter recruiting to toll-like receptor 4 TICAM-1 that induces interferon-beta. *J Biol Chem.* (2003) 278:49751–62. doi: 10.1074/jbc.M305820200
27. Bazzoni G. Endothelial tight junctions: permeable barriers of the vessel wall. *Thromb Haemost.* (2006) 95:36–42. doi: 10.1160/TH05-07-0488
28. Foxman EF, Campbell JJ, Butcher EC. Multistep navigation and the combinatorial control of leukocyte chemotaxis. *J Cell Biol.* (1997) 139:1349–60. doi: 10.1083/jcb.139.5.1349
29. Kagari T, Doi H, Shimozato T. The importance of IL-1 beta and TNF-alpha, and the noninvolvement of IL-6, in the development of monoclonal antibody-induced arthritis. *J Immunol.* (2002) 169:1459–66. doi: 10.4049/jimmunol.169.3.1459
30. Pastor CM, Rubbia-Brandt L, Hadengue A, Jordan M, Morel P, Frossard JL. Role of macrophage inflammatory peptide-2 in cerulein-induced acute pancreatitis and pancreatitis-associated lung injury. *Lab Invest.* (2003) 83:471–8. doi: 10.1097/01.LAB.0000063928.91314.9F
31. Kollmar O, Menger MD, Schilling MK. Macrophage inflammatory protein-2 contributes to liver resection-induced acceleration of hepatic metastatic tumor growth. *World J Gastroenterol.* (2006) 12:858–67. doi: 10.3748/wjg.v12.i6.858
32. Deshmane SL, Kremlev S, Amini S, Sawaya BE. Monocyte chemoattractant protein-1 (MCP-1): an overview. *J Interferon Cytokine Res.* (2009) 29:313–26. doi: 10.1089/jir.2008.0027
33. Mei J, Liu Y, Dai N, Favara M, Greene T, Jeyaseelan S, et al. CXCL5 regulates chemokine scavenging and pulmonary host defense to bacterial infection. *Immunity.* (2010) 33:106–17. doi: 10.1016/j.immuni.2010.07.009
34. Kim MH, Chang HM, Kim TW, Lee SK, Park JS, Kim YH, et al. EC-18, a synthetic monoacetyldiacylglyceride, inhibits hematogenous metastasis of KIGB-5 biliary cancer cell in hamster model. *J Korean Med Sci.* (2009) 24:474–80. doi: 10.3346/jkms.2009.24.3.474
35. Hong JJ, Koh Y, Park JS, Jung HD, Kim SH, Lee TS, et al. Enteral administration of a synthetic monoacetyldiglyceride improves survival in a murine model of abdominal sepsis. *J Trauma.* (2010) 68:62–8. doi: 10.1097/TA.0b013e3181c3fede
36. Shin IS, Shin NR, Jeon CM, Kwon OK, Sohn KY, Lee TS, et al. EC-18, a synthetic monoacetyldiglyceride (1-palmitoyl-2-linoleoyl-3-acetylglycerol), attenuates the asthmatic response in an aluminum hydroxide/ovalbumin-induced model of asthma. *Int Immunopharmacol.* (2014) 18:116–23. doi: 10.1016/j.intimp.2013.11.006
37. Lewis AJ, Seymour CW, Rosengart MR. Current Murine Models of Sepsis. *Surg Infect.* (2016) 17:385–93. doi: 10.1089/sur.2016.021
38. Seemann S, Zohles F, Lupp A. Comprehensive comparison of three different animal models for systemic inflammation. *J Biomed Sci.* (2017) 24:60. doi: 10.1186/s12929-017-0370-8
39. Matute-Bello G, Frevert CW, Martin TR. Animal models of acute lung injury. *Am J Physiol Lung Cell Mol Physiol.* (2008) 295:L379–99. doi: 10.1152/ajplung.00010.2008
40. Shin IS, Ahn KS, Shin NR, Lee HJ, Ryu HW, Kim JW, et al. Protective effect of EC-18, a synthetic monoacetyldiglyceride on lung inflammation in a murine model induced by cigarette smoke and lipopolysaccharide. *Int Immunopharmacol.* (2016) 30:62–8. doi: 10.1016/j.intimp.2015.11.025

Conflict of Interest Statement: H-RL, SY, and K-YS were employed by company, ENZYCHEM Lifesciences (Bio valley-ro, Jechon-si, Chungcheongbuk-do, Republic of Korea).

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Advantages of publishing in Frontiers

OPEN ACCESS
Articles are free to read for greatest visibility and readership

FAST PUBLICATION
Around 90 days from submission to decision

HIGH QUALITY PEER-REVIEW
Rigorous, collaborative, and constructive peer-review

TRANSPARENT PEER-REVIEW
Editors and reviewers acknowledged by name on published articles

Frontiers
Avenue du Tribunal-Fédéral 34
1005 Lausanne | Switzerland

Visit us: www.frontiersin.org
Contact us: info@frontiersin.org | +41 21 510 17 00

REPRODUCIBILITY OF RESEARCH
Support open data and methods to enhance research reproducibility

DIGITAL PUBLISHING
Articles designed for optimal readership across devices

FOLLOW US
@frontiersin

IMPACT METRICS
Advanced article metrics track visibility across digital media

EXTENSIVE PROMOTION
Marketing and promotion of impactful research

LOOP RESEARCH NETWORK
Our network increases your article's readership