

FcεRI stimulation promotes the differentiation of histamine receptor 1-expressing inflammatory macrophages

N. Novak^{1,*}, W. M. Peng^{1,*}, T. Bieber¹ & C. Akdis²

¹Department of Dermatology and Allergy, University of Bonn Medical Center, Bonn, Germany; ²Swiss Institute of Allergy and Asthma Research (SIAF), University of Zurich, Davos, Switzerland

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Correspondence

Prof. Dr. Natalija Novak, Department of Dermatology and Allergy, University of Bonn, Sigmund-Freud-Str. 25, 53105 Bonn, Germany.

Tel.: +49-228-287-15370

Fax: +49-228-287-14333

E-mail: Natalija.Novak@ukb.uni-bonn.de

*These authors contributed equally to this study.

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Abstract

Background: Monocyte differentiation into dendritic cells or macrophages and recruitment to peripheral organs in chronic inflammatory diseases are directed by allergen challenge via FcεRI as well as the nature of soluble factors in the micro-environment. High-affinity receptor for IgE stimulation of effector cells results in the release of histamine, which acts on various histamine receptors (HR) 1–4, expressed by immune cells.

Methods: We examined the effect of FcεRI stimulation of human monocytes on H1R expression and function of differentiating cells. The mRNA levels of H1R, H2R and histidine decarboxylase of differentiating cells were detected by quantitative real-time PCR. Expression of CD1c, CD11c, CD68 and CD163 was detected by flow cytometry. Amount of histamine, IL-6 and IL-12p70 in the cell culture was measured with the help of cytometric bead arrays or ELISA assays. Numbers of H1R-expressing macrophages were evaluated by immunofluorescence double staining of CD68 and H1R on human skin sections.

Results: We demonstrated that FcεRI stimulation promotes the generation of H1R-expressing macrophage-like cells with enhanced histamine biosynthesis and H1R-mediated proinflammatory properties. Supporting our *in vitro* findings, high numbers of H1R-expressing CD68^{pos} macrophages were detected in the dermis of atopic dermatitis (AD) skin lesions.

Conclusion: Our observations point to a close histamine-/HR-mediated activation of dermal macrophages, leading to modified cell differentiation and responsiveness via H1R, which might contribute to the aggravation of allergic skin inflammation in AD.

The effects of allergen exposure in allergic inflammatory diseases such as atopic dermatitis (AD) are mediated by high-affinity receptor for IgE (FcεRI)-bearing mast cells on one hand and FcεRI^{pos} antigen-presenting cells (APCs) on the other hand (1). Stimulation of FcεRI on mast cells leads to the release of soluble factors such as histamine. Histamine,

one of the most important mediators of allergic inflammatory reactions, is a dibasic multifunctional amine produced by the help of histidine decarboxylase (HDC) (2). Histamine is expressed in lung, skin and gastrointestinal tract and is responsible for vasodilatation, smooth muscle contraction or oedema formation. Further on, histamine is not only involved in allergic immune responses of the immediate type, but plays a key role in allergic inflammatory reactions (3–7). In the skin, histamine released by mast cells acts preferentially on dermal dendritic cells (DCs) and macrophages (MΦ) via histamine receptors (HRs) expressed by these cells. So far, four different human HR types have been identified (H1R–4) and the expression pattern of HRs is decisive for

Abbreviations

DC, dendritic cell; FcεRI, high-affinity receptor for IgE; HDC, histidine decarboxylase; HR, histamine receptor; Mo, monocyte; MoDCs, monocyte-derived dendritic cells; MoMΦ, monocyte-derived macrophages; MΦ, macrophage.

the nature of the outcoming immune response (6, 8). H1R owns much proinflammatory and cell-activating properties, while H2R has been shown to be involved in some tolerogenic immune responses evolving from H2R-mediated suppressive effects (3). During the differentiation of monocytes into MΦ (MoMΦ), H1R is upregulated and H2R is downregulated; in contrast, H1R is downregulated and H2R is upregulated when cells develop into monocyte-derived DCs (MoDCs) (9, 10). H1R activates specific G-proteins, and G-protein-coupled signalling involves phospholipase C activation, inositol phosphate production and intracellular Ca²⁺ flux (8) as well as the activation of NF-κB (11), STAT1 (12), STAT4 (13) and mitogen-activated protein kinase (MAPK) (14) pathways. Furthermore, H1R stimulation increases the release of interleukin (IL)-6 and β-glucuronidase of lung MΦs (15, 16). Despite mast cells and basophils, some leucocyte subpopulations such as DCs and MΦs are capable of producing and releasing histamine as well (17, 18).

Increased plasma and skin levels of histamine have been observed in patients with AD (19), and mast cells present in the dermal compartment have been suggested as major source (20). Allergen uptake, processing and presentation to T cells is a central mechanism in AD, which is supposed to be accomplished mainly by APCs such as monocytes or DCs in the skin and in the blood, which are equipped with respective receptors including FcεRI (21). Notably, a close interaction of antigen receptor-mediated immune responses and signalling with G-coupled H1R was observed in murine models, implying that comparable coregulatory functions of FcεRI and H1R might exist (22). In this study, we examined the effect of FcεRI stimulation of human monocytes on the H1R expression as well as responsiveness of H1R of differentiating cells. We demonstrate that FcεRI stimulation promotes the generation of H1R-expressing MΦs with high proinflammatory properties and increased histamine biosynthesis. In correlation with our *in vitro* findings, H1R-expressing CD68^{pos} MΦs were detectable in high amounts in the dermal compartment of AD skin lesions. Our observations point to a close histamine-/H1R-mediated activation of MΦs, which might follow allergen challenge in allergic inflammatory diseases such as AD.

Methods

Reagents

Rabbit anti-human IgE and unconjugated mAb against CD68 (mIgG3, clone: PG-M1) were purchased from Dako (Glostrup, Denmark). Monomeric human myeloma IgE (hIgE) was from Calbiochem-Novabiochem Corp. (San Diego, CA, USA). FITC-conjugated goat-anti-mouse IgG (GaM/FITC) was from Jackson Immuno-Research Laboratories (West Grove, PA, USA). Rabbit monoclonal anti-CD1a (IgG1, clone: EP3622) antibody was from Abcam (Cambridge, MA, USA). Monoclonal antibodies (mAbs) against CD68 (mIgG2b, clone: Y1/82A), CD11c (mIgG1κ, clone: B-ly6), and allophycocyanin (APC)-labelled mAb against CD14 (mIgG2a, clone: M5E2) were from BD Biosciences Pharmingen (Heidelberg, Germany); unconjugated mAb against

CD163 (mIgG1, clone: 5C6-FAT) was from Acris (Herford, Germany); unconjugated mAb against CD54 (mIgG1, clone: 84H10) was from Immunotech (Marseille Cedex, France); polyclonal rabbit anti-H1R antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA); unconjugated mAb against CD1c (mIgG1, clone: L161) and normal mouse serum for blocking purposes were obtained from Dianova (Hamburg, Germany); and PE-labelled T6RD1 (mIgG1) mAb from Beckman Coulter (Krefeld, Germany) was directed against CD1a. All other reagents were obtained from Sigma Aldrich (Taufkirchen, Germany) unless otherwise indicated.

Preparations and cultures

After the written informed consent and local ethical committee approval were obtained, the blood for monocyte isolation was taken from allergic volunteers at the Department of Dermatology and Allergy of the University of Bonn. Monocytes were isolated from peripheral blood as described before (23) and cultured for 6 days in very low endotoxin medium RPMI 1640 (Biochrom, Berlin, Germany) with 1% antibiotic and antimycotics, 10% inactivated foetal calf serum (FCS) with 500 U (53.82 ng)/ml IL-4 (Gentaur GmbH, Aachen, Germany) and 500 U (89.28 ng)/ml GM-CSF (Berlex Laboratories, Inc., Richmond, CA, USA).

Receptor stimulation

Stimulation of FcεRI was achieved as previously described (23). Briefly, cells were harvested and washed with culture medium twice, and then incubated with 1 µg/ml hIgE for 1 h at 37°C. After washing with culture medium, 10 µg/ml anti-human IgE was added to perform aggregation of FcεRI-bound IgE.

Cell surface and intracellular phenotyping of MoDCs/MoMΦs by flow cytometry

After 6 days in culture, intercellular and extracellular staining was performed as reported in detail elsewhere (24). Briefly, flow cytometry was performed using a FACS-Canto (BD Biosciences) and analysed by FACSDiva (BD Biosciences) and FlowJo (TreeStar Inc., Ashland, OR, USA) software.

Measurement of histamine, IL-6 and IL-12p70 in the cell culture supernatant

At day 6 of culture, MoDC/MoMΦ were left unstimulated or stimulated with betahistidine 10⁻⁴ M or histamine 10⁻⁴ M for 6 h to determine IL-6 production and with betahistidine 10⁻⁴ M or histamine 10⁻⁴ M together with interferon (IFN)-γ (200 U/ml; R&D systems, Wiesbaden, Germany) and poly I:C (Sigma Aldrich) 10 µg/ml for 24 h to determine IL-12p70 secretion. Supernatants were collected in separator tubes, centrifuged for 10 min at approximately 1000 g and stored at -20°C. IL-6 and IL-12p70 in the supernatant were detected by cytokine bead array from BD Biosciences following instruction manual. To determine histamine secretion of differentiating cells, at day 1 of culture cells were simulated

for 30 min with LPS (10 ng/ml; Sigma Aldrich) and the cell culture supernatant was analysed with the help of ELISA assay (Neogen Corporation, Scotland, UK) as described in the instruction manual.

Real-time PCR

mRNA was isolated with the help of the RNeasy Mini kit (Qiagen, Hilden, Germany) including digestion of genomic DNA and was subjected to cDNA synthesis with TaqMan reverse transcription reagents with random hexamers according to the manufacturer's instructions (Applied Biosystems, Darmstadt, Germany). The prepared cDNA was amplified using TaqMan assay Master Mix (Applied Biosystems) according to the recommendations of the manufacturer in an ABI Prism 7300 Sequence Detection System (Applied Biosystems). Primers were as follows: H1R forward primer 5'-TCT CGA ACG GAC TCA GAT ACC A-3', H1R reverse primer 5' CCT GTG TTA GAC CCA CTC CTC AA-3', H1R probe FAM-ACA GAG ACA GCA CCA GGC AAA GGC AA-TAMRA; H2R forward primer 5'-GCT GGG CTA TGC CAA CTC A-3', H2R reverse primer 5'-GGT GCG GAA GTC TCT GTT CAG-3', and H2R probe FAM-CCC TGA ACC CCA TCC TGT ATG CTG C-TAMRA (all from Microsynth AG, Balgach, Switzerland); HDC (Hs00157914_m1) as well as endogenous controls (18) were from Applied Biosystems. All assays were performed according to the manufacturer's instructions. Relative quantification and calculation of the range of confidence were performed using the comparative $\Delta\Delta C_T$ methods (25). All analyses were conducted in duplicates.

Immunofluorescence staining of skin sections

Immunofluorescence staining was performed on 4- μ m skin sections of paraformaldehyde-fixed and paraffin-embedded

skin biopsies as described elsewhere (26). Sections were treated with monoclonal antibody against CD68 (Dako) at 4°C overnight. Expression of CD1a or H1R was analysed using anti-CD1a (Abcam) or anti-H1R antibody (Santa Cruz Biotechnology). Appropriate isotype-matched IgG (Sigma Aldrich) were used as experimental antibody controls. Antibody binding was detected with anti-mouse and anti-rabbit Cy-2- and Cy-3-labelled secondary antibodies (Jackson Immuno-Research Laboratories). Sections were analysed with a fluorescence microscope (Leica Microsystems, Wetzlar, Germany) using Diskus and ImageJ software (National Institute of Health website, <http://rsbweb.nih.gov/ij/>) for documentation and analysis.

Statistical analysis

Statistical analysis was performed with SPSS 17.0 for Windows (SPSS, Chicago, IL, USA). Quantitative values were compared by nonparametric testing (Wilcoxon). Results are given in mean $\pm$ standard error of the mean (SEM), respectively. Any *P*-values are two-sided and subject to a global significance level of 5%.

Results

FcεRI stimulation promotes the differentiation of H1R-expressing cells

Stimulation of FcεRI on human monocytes shifts the differentiation of MoDCs towards MΦs including high capacity of phagocytosis and CD14 expression (23). H1R expression has been reported to be upregulated during differentiation of MoMΦs, while expression of H2R is downregulated (9, 10). We observed that stimulation of FcεRI on monocytes upregulates H1R mRNA expression already after 24 h (Fig. 1A). As most of the donors were atopic, constitutive

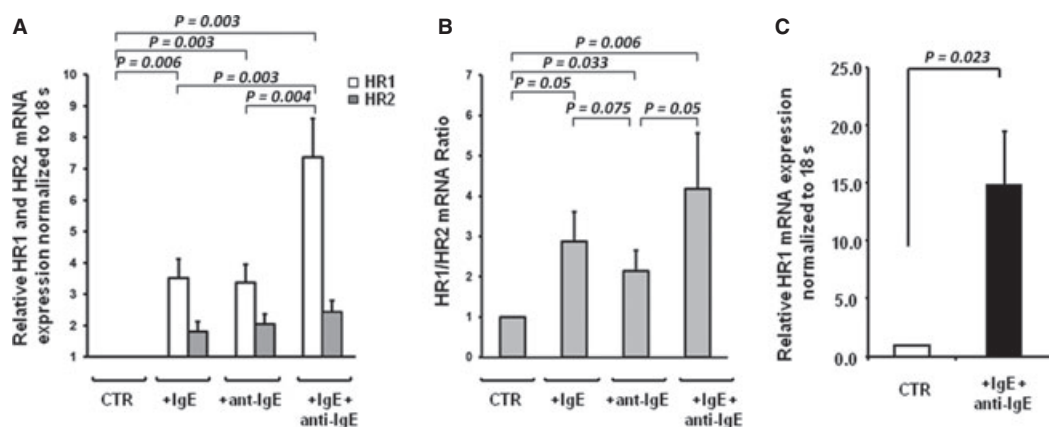


Figure 1 Stimulation of FcεRI mediates elevated H1R mRNA expression of differentiating cells. (A) Relative mRNA expression of H1R and H2R of unstimulated monocytes (CTR), cells incubated with IgE alone for 1 h (+IgE), stimulated with anti-IgE alone (+ anti-IgE) or preincubated with IgE, washed and cross-linked with anti-IgE (+IgE/anti-IgE) at the onset of *in vitro* culture is depicted, and (B) ratio of relative mRNA

expression of H1R/H2R is calculated; mean value $\pm$ standard error of the mean (SEM) of *n* = 11 experiments is shown. (C) Cells were cultured for 6 days. Relative expression of H1R mRNA of the cells on day 6 of culture of unstimulated (IgE-R(−)) monocytes and monocytes activated via FcεRI (IgE-R(+)); mean value $\pm$ standard error of the mean (SEM) of *n* = 8 experiments is shown.

IgE bound to FcεRI stimulated by addition of anti-IgE alone was sufficient to significantly upregulate the H1R mRNA level, while saturation of the remaining FcεRI binding sites with exogenously added IgE prior to FcεRI stimulation led to a further increase in H1R mRNA expression. H2R mRNA expression was slightly increased, resulting in an increased H1R/H2R mRNA ratio (Fig. 1B). Higher H1R mRNA expression of FcεRI-preactivated cells was sustained during *in vitro* culture, and elevated H1R mRNA expression of FcεRI-activated cells was also observed on day 6 of culture (Fig. 1C).

FcεRI stimulation induced H1R-expressing cells express surface marker of MΦs

As a next step, we evaluated the expression pattern of surface markers of MΦs and dermal DCs of cells differentiated in the presence of IL-4 and GM-CSF from FcεRI-stimulated monocytes as compared to cells derived from unstimulated control monocytes. With the help of double staining of CD14 and CD1a, we observed that unstimulated human monocytes mainly differentiated into CD1a^{pos} MoDCs, while stimulation of FcεRI promoted the differentiation of an additional fraction of CD14^{pos} cells besides CD1a^{pos} MoDCs (Fig. 2A). Furthermore, we evaluated expression of CD1c, CD11c, CD54, CD68 and CD163 on CD1a^{pos} and CD14^{pos} cells. CD14^{pos} cells expressed less CD1c and CD11c and more CD68 and CD163 than CD1a^{pos} cells (Fig. 2A,B). From these data, we conclude that part of the cells differentiate into MΦs after FcεRI stimulation.

FcεRI stimulation induced H1R-expressing cells display characteristics of MΦs with high IL-6 and low IL-12p70 releasing properties

Furthermore, we analysed the cytokine profile of the differentiating cells. Cells generated from FcεRI-stimulated precursors secreted more proinflammatory cytokine IL-6 (Fig. 3A). Secretion of IL-6 further increased significantly after stimulation of H1R with the H1R-agonist betahistidine or stimulation of the cells with histamine. In contrast, cells generated after FcεRI stimulation released significantly lower amounts of IL-12p70, which is secreted by DCs and promotes immune responses of the Th1 type (Fig. 3B). From these data, we conclude that FcεRI stimulation leads to the differentiation of MΦs with high secretion of the proinflammatory cytokine IL-6 and lower production of IL-12p70.

FcεRI-stimulated proinflammatory MΦs display increased histamine biosynthesis

Increased biosynthesis of histamine indicated by higher HDC mRNA expression during differentiation of DCs as well as by murine MΦs and increased histamine release after stimulation of DCs with LPS has been demonstrated in previous studies (17, 18). We wanted to know next whether FcεRI stimulation modifies endogenous histamine synthesis of the differentiating cells. Therefore, mRNA of FcεRI-stimulated and unstimulated cells was isolated at days 1 and 6 of culture. We observed significantly upregulated HDC mRNA levels on both day 1 (Fig. 4A) and day 6 (Fig. 4B) in cells,

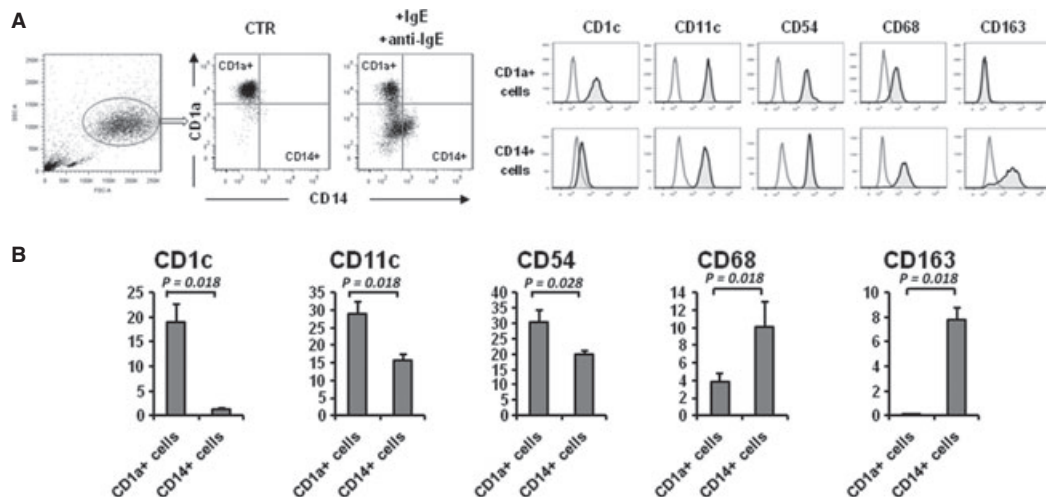


Figure 2 Stimulation of FcεRI promotes human monocytes to differentiate into MoMΦs. (A) Freshly isolated human monocytes from allergic donors were isolated and prestimulated via FcεRI. After 6 days of culture, cells were labelled with PE-conjugated antibody against CD1a and APC-conjugated antibody against CD14, and expression levels of CD1c, CD11c, CD54, CD68 and CD163 on CD14^{pos} and CD1a^{pos} cells were evaluated by flow cytometry. Surface expression of CD1c, CD11c, CD54 and CD163 and intracellular

expression of CD68 in CD1a^{pos} and CD14^{pos} cells were depicted as representative overlay histogram. Isotype-matched IgG control antibodies were used as a control. Isotype control (grey line), respective marker (black line). One representative experiment of $n = 7$ is shown (A). The expression levels of CD1c, CD11c, CD54, CD68 and CD163 on both CD1a^{pos} and CD14^{pos} cells are depicted as relative fluorescence index (rFI). Mean + SEM for $n = 7$ is shown.

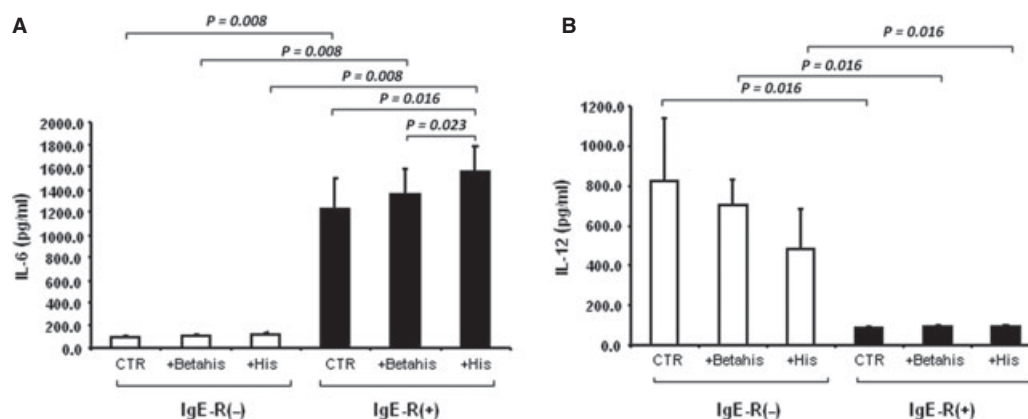


Figure 3 Higher IL-6 secretion and lower IL-12 production of FcεRI prestimulated cells. Human monocytes were cultured with (IgE R (+)) or without (IgE R(-)) FcεRI prestimulation after 6 days of culture; cells were stimulated as described in Methods. Amount of

the pro-inflammatory cytokine IL-6 ($n = 7$) and the Th1 immune responses promoting cytokine IL-12 ($n = 6$) in the cell culture supernatant was evaluated by cytometric bead array quantification. Mean value + SEM is depicted.

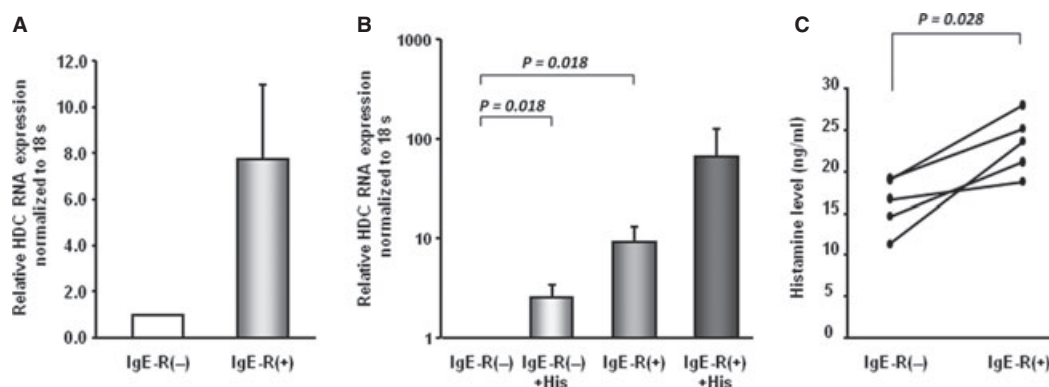


Figure 4 FcεRI stimulation increases histamine biosynthesis of differentiating cells. Relative histidine decarboxylase (HDC) mRNA expression of unstimulated and FcεRI-stimulated cells at day 1 ($n = 5$) (A) and day 6 ($n = 7$) (B) of *in vitro* culture is shown.

Amount of histamine in the supernatant of unstimulated and FcεRI-stimulated MoDCs/MoMΦs released after 30 min of incubation with LPS is depicted as mean value (C).

which have been stimulated via FcεRI at the onset of the culture. Interestingly, exogenous histamine at day 1 of cultures further increased HDC mRNA expression of the cells (Fig. 4B). In parallel, higher amounts of histamine were detectable in the cell culture supernatant of FcεRI-stimulated cells stimulated concomitantly with LPS to induce histamine secretion as compared to unstimulated control cells incubated with LPS (Fig. 4C). These data demonstrate that FcεRI stimulation as well as histamine in the cellular microenvironment amplifies the endogenous histamine biosynthesis of differentiating cells.

High numbers of H1R-expressing MΦs are located in the dermis of atopic dermatitis lesions

Infiltration of AD lesions with distinct DC subtypes and MΦ has been demonstrated (27). To evaluate the *in vivo* relevance of our findings, paraffin-embedded skin sections taken from lesional skin of patients with AD and healthy controls were

analysed by immunofluorescence staining. Double staining of skin sections demonstrated that CD1a^{pos} DCs exist in epidermis while CD68^{pos} MΦs were mainly located in the dermis (Fig. 5A). Infiltrates of H1R-expressing cells with morphologic characteristics of MΦs expressing CD68 were detectable in the upper part of the dermis as well as the lower dermal compartment (Fig. 5B) of lesional skin from patients with AD. In contrast, only few H1R^{pos}CD68^{pos} MΦs were detectable in sections taken from healthy skin.

Discussion

FcεRI-mediated allergen challenge and soluble factors in the microenvironment determine the nature of the differentiating cells and their capacity, to amplify the allergic inflammatory immune response. Here, we demonstrate that FcεRI stimulation of human monocytes promotes the differentiation of H1R-expressing MΦs with increased production of proinflammatory cytokines.

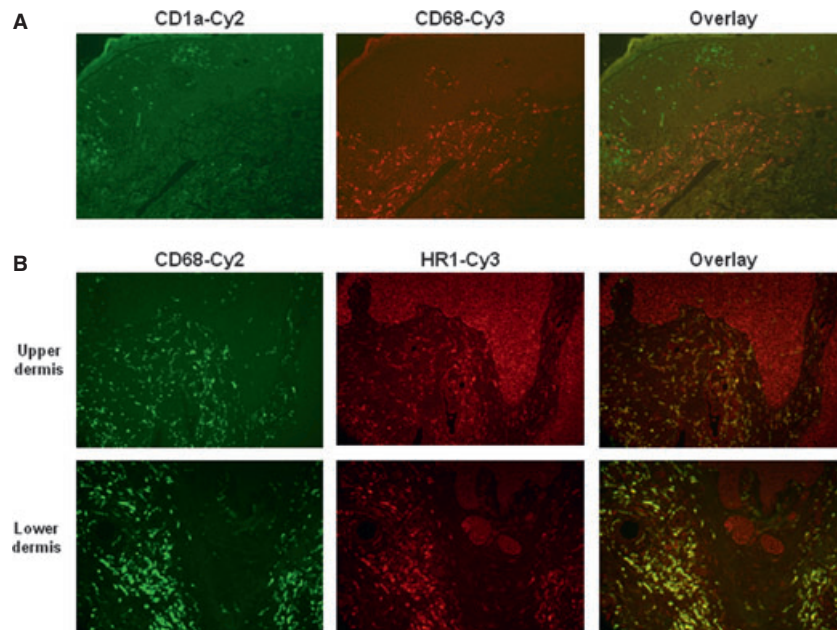


Figure 5 H1R/CD68 double-positive MΦs are present in AD skin. Representative immunofluorescence double staining of a skin biopsy taken from lesional skin from patients with AD ($n = 2$) is shown. Skin sections stained with anti-CD1a antibody labelled with Cy-2 (green) and anti-CD68 antibody labelled with Cy-3 (red) as well as the overlay of both stainings (yellow) are depicted (A).

Representative immunofluorescence double staining of a skin biopsy taken from lesional skin from patients with AD is shown. Skin section stained with an anti-CD68 antibody labelled with Cy-2 (green) and an anti-H1R antibody labelled with Cy-3 (red) as well as the overlay of both stainings (yellow) is depicted (B).

FcεRI stimulation and efficient allergen uptake and presentation to T cells are central mechanisms of allergic diseases. Stimulation of FcεRI on monocytes promotes NF-κB signalling (28), prevents Fas/Fas-L-mediated apoptosis (29), induces the production of IL-10 (23), upregulates the expression of indoleamine dioxygenase (IDO) and favours the release of proinflammatory mediators such as IL-6, monocytes chemoattractant protein (MCP)-1 or macrophage inflammatory protein (MIP)-1β (30). In addition, FcεRI stimulation and consequent IL-10 secretion profoundly promote the differentiation of MΦ, while preventing the differentiation of CD1a^{pos} DCs (23). Our data provide evidence for a close interaction and regulation of FcεRI with HRs, in particular H1R, and the sensitivity of differentiating cells for H1R-mediated signals during allergic immune responses.

As we could show that FcεRI stimulation further increases histamine biosynthesis of the differentiating cells by directly upregulating mRNA expression of HDC, an endogenous feedback amplification consisting of FcεRI-mediated upregulation of H1R, enhanced autologous supply of histamine and thereby increased H1R mediated signalling is very likely. Endogenous histamine synthesis and HDC activity are assumed to play a crucial role during the differentiation and proliferation of cells. As a proof of this concept, blockage of HDC profoundly disturbs DC differentiation (18). This implies that the modified HDC expression induced by FcεRI stimulation might in part further impact on the altered cell differentiation observed here. *In vivo*, the autologous supply

of histamine by differentiating cells itself might be replenished by histamine provided by mast cells in the dermis, which undergo frequent allergen challenge in allergic diseases such as AD.

MΦs are regarded as important immune cells for a long time and their activities are grouped into three main functions: host defence, wound healing and immune regulation (31). Infiltration of MΦs is regarded as a hallmark of inflammatory skin diseases such as psoriasis and AD (32). It is assumed that monocytes recruited from the blood during inflammation represent an important source for inflammatory MΦs in the peripheral tissue (31). MΦs and DCs are outlined in the mononuclear phagocyte systems (MPS) and derive from a common progenitor that allows to some degree the recapitulation of the differentiation from DCs to MΦs or back through the modification of environmental cytokines and growth factors (33). High H1R expression has been described in human *in vitro*-generated MoMΦs as well as human lung MΦs of asthmatics (9). Histidine decarboxylase-expressing CD68^{pos} MΦs are detectable at chronic inflammatory sites such as in human atherosclerotic carotid arteries (34), indicating that this subset of MΦs is actively involved in the maintenance of allergic and inflammatory immune responses. Infiltrates of CD68^{pos} MΦs are detectable in the epidermal, but foremost in the dermal part of lesional AD skin, but there is not much knowledge about the origin of these cells as well as specific mechanisms boosting their proinflammatory properties

within the skin. Here, we provide a model of enhanced differentiation and activation of H1R-expressing MΦs regulated by the degree of FcεRI stimulation of their monocytic precursor cells as well as mast cells in their microenvironment.

Concerning the high density and close contact of mast cells and H1R-bearing MΦs in the dermal compartment of the skin in AD or the airways of asthmatic patients (9), a cross-talk of mast cells and MΦs via histamine as a mediator represents a conceivable *in vivo* scenario. Hence, the augmentation of antigen-receptor-mediated responses by H1R such as shown here might directly account for the amplification of the allergic inflammatory reaction *in vivo* and represent therefore a target for therapeutic intervention. So far, application of H1R antagonists for the treatment of AD has been only of limited value as most of the studies focused on the antipruritic effect of H1R antagonists in AD (35–37). However, there are also studies that support the concept that H1R antagonists, although being not beneficial in regard to pruritus, might nevertheless ameliorate AD, mirrored by a steroid-sparing effect achievable under treatment, which indirectly provides evidence for positive effects of some antihistamines on skin inflammation (38). Taken together, in view of the data presented here, FcεRI-H1R cross-regulated mechanisms impacting on MΦ differentiation and activation might aggravate

skin inflammation in AD and should be therefore considered in the development of novel therapeutic strategies.

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Authors' contributions

N.N., C.A. and W.M.P. contributed to experimental design. N.N. and W.M.P. performed the experiments, data analysis, interpretation of the results. N.N., W.M.P. and C.A. wrote the manuscript. T.B. reviewed the manuscript. N.N. gained funding.

Conflict of interest

The authors have no conflicting financial interests.

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