The Histamine H_4 Receptor Is Highly Expressed on Plasmacytoid Dendritic Cells in Psoriasis and Histamine Regulates Their Cytokine Production and Migration

Maria Gschwandtner[1,2], Susanne Mommert[1], Brigitta Köther[1], Thomas Werfel[1] and Ralf Gutzmer[1]

Plasmacytoid dendritic cells (pDC) are present in inflammatory skin lesions, in particular, in psoriasis. In such lesions, the inflammatory mediator histamine is also detected in high amounts. We therefore investigated a possible interaction of pDC with histamine, especially via the most recently described histamine H_4 receptor (H_4R). We detected the expression of the H_4R on pDC in the blood and in lesional psoriasis skin. Interestingly, compared with healthy controls and patients with atopic dermatitis, pDC from the blood of psoriasis patients expressed the highest levels of the H_4R, which was even more upregulated on stimulation with IFN-γ and CpG. After activation of the H_2R and H_4R on pDC, we observed downregulation of CpG-induced production of tumor necrosis factor α, IFN-α, and CXCL8, but not of the chemokine CXCL10. Histamine-induced downregulation of cytokine production was more pronounced in pDC derived from psoriasis patients. Furthermore, we observed F-actin polymerization and active migration of pDC in response to H_4R agonist stimulation. Taken together, our results indicate that the H_4R is highly expressed on pDC in psoriasis and influences cytokine production and migration of pDC. Therefore, the H_4R alone or in combination with the H_2R might be a promising therapeutic target in psoriasis.

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INTRODUCTION

Various types of dendritic cells (DC) have an important role in inflammatory skin diseases. Among them plasmacytoid dendritic cell (pDC) which represent a rare dendritic cell (DC) sub-population characterized by plasma cell-like shape and the surface marker phenotype $CD4^+$, $CD45RA^+$, $CD123^+$ (also known as IL-3Rα), BDCA-2^+, BDCA-4^+, $ILT3^+$, and $CD11c^-$, $lineage^-$ (Colonna *et al.*, 2004). In contrast to other DC subtypes, pDC do not express the toll-like receptors (TLR) 2–5 on their surface, but they express TLR7 and TLR9 in their endosomal compartments (Hochrein *et al.*, 2002). In response to stimulation of these innate receptors with single-stranded RNA and unmethylated CpG oligodeoxynucleotides, pDC have the unique capacity to produce massive amounts of type I interferons and other proinflammatory cytokines, such as tumor necrosis factor (TNF)-α, CXCL10, and CXCL8 (Hochrein *et al.*, 2002; Ito *et al.*, 2004). pDCs are of particular importance in antiviral immunity; however, by their cytokine production they can influence immune responses in general, by modulating the function of T cells, antigen-presenting cells and keratinocytes (Colonna *et al.*, 2004).

pDC typically reside in the peripheral blood and are absent in healthy skin, but they were shown to infiltrate lesions of inflammatory skin diseases, such as lupus erythematosus, allergic contact dermatitis, atopic dermatitis, and psoriasis (Wollenberg *et al.*, 2002; Bangert *et al.*, 2003; Stary *et al.*, 2005). Especially in psoriasis pDCs seem to be of pathogenetic relevance as pDCs are recruited to the skin and their activation results in high-level secretion of IFN-α. One key factor activating pDC in psoriasis is LL37, an antimicrobial peptide abundant in psoriasis. LL37 was shown to be involved in the conversion of self-DNA into danger signal, which is recognized by pDC and thereby drives autoimmunity in psoriasis (Lande *et al.*, 2007). Moreover, it was shown that psoriasis patients have increased serum levels of pDC-derived cytokines correlating with the severity of psoriasis (Arican *et al.*, 2005). These findings provide good evidence that pDCs are involved in the pathology of psoriasis; however, the exact mechanism how pDC migrate to the inflamed skin in psoriasis and how their function is regulated in disease is not elucidated in detail yet.

[1]*Division of Immunodermatology and Allergy Research, Department of Dermatology and Allergy, Hannover Medical School, Hannover, Germany*

Correspondence: Maria Gschwandtner, Division of Immunodermatology and Allergy Research, Department of Dermatology and Allergy, Hannover Medical School, Carl-Neuberg-Street 1, D-30625 Hannover, Germany. E-mail: maria_gschwandtner@gmx.at

[2]*Current address: Department of Dermatology, Medical University of Vienna, Vienna, Austria.*

Abbreviations: 4-MH, 4-methylhistamine; CpG, CpG oligonucleotide ODN2216; DC, dendritic cells; H_xR, histamine receptor; pDC, plasmacytoid dendritic cells; PMBC, peripheral blood mononuclear cells; TLR, toll-like receptor

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As histamine is also present in high amounts in lesions of psoriasis (Krogstad *et al.*, 1997) and therefore comes into contact with pDC, we decided to investigate the functional role of histamine on pDC. Histamine can bind to four different seven-transmembrane histamine receptors (H_xR), which are signaling through coupled G-proteins and have diverse functions in physiological and pathological processes. The H_1R, H_2R, and H_4R have a role as regulators of immune responses, the H_2R is involved in gastric acid secretion and the H_3R is important for neurotransmission. Concerning the function of histamine on immune cells several studies have been performed, showing that histamine affects the migration behavior and mediator secretion of T cells (Gutzmer *et al.*, 2009), monocytes (Dijkstra *et al.*, 2007), eosinophils (Ling *et al.*, 2004), and keratinocytes (Giustizieri *et al.*, 2004; Gschwandtner *et al.*, 2008). Regarding the role of histamine on pDC a previous study showed histamine-mediated downregulation of TNF-α and IFN-α production (Mazzoni *et al.*, 2003), which was in part attributed to the H_2R. However, this study did not take into account the most recently discovered H_4R (Nakamura *et al.*, 2000), which is of particular importance in the regulation of immune cells (Zampeli and Tiligada, 2009). Therefore, we hypothesized that the H_4R has a role in pDC biology and, hence, we performed detailed analysis of the expression, regulation and function of the H_4R on pDC, especially in the context of the inflammatory skin disease psoriasis.

RESULTS

High expression of the H_4R on pDC in skin and blood of psoriasis patients

The expression of the H_4R and other histamine receptors on isolated pDC from buffy coats was tested at the mRNA level by LightCycler real-time PCR. Although we could regularly detect mRNA for H_1R, H_2R, and H_4R, but we could not detect mRNA for the H_3R on pDC (Figure 1).

To further evaluate H_4R expression *in situ,* we performed immunofluorescence staining in skin samples from psoriasis patients, in which the infiltration of pDC has been described previously (Wollenberg *et al.*, 2002; Nestle *et al.*, 2005). As depicted in Figure 2, we detected double-positive pDC expressing the H_4R in the dermis of psoriasis patients.

To analyze H_4R expression on the protein level in blood pDC, peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood and pDCs were identified by cell surface staining of CD123 and absence of lineage staining (CD3, CD14, CD16, and CD19). Intracellular staining of the gated pDC showed that pDC express the H_4R on the protein level (Figure 3a). We analyzed H_4R expression levels on pDC derived from individuals without inflammatory skin diseases, patients with atopic dermatitis and patients with psoriasis and observed significantly higher H_4R expression on pDC derived from patients with psoriasis (Figure 3b).

Cytokine regulation of H_4R expression

The regulation of H_4R expression on pDC was investigated after stimulation with the Th1-associated cytokine IFN-γ, the TLR9 ligand CpG, and the Th2 cytokine IL-13. We observed significant upregulation of the H_4R upon IFN-γ and CpG stimulation on pDC derived from psoriasis patients, whereas IL-13 had no effect (Figure 3c). In pDC derived from patients with atopic dermatitis and healthy control subjects, we did not observe H_4R regulation with any of the used cytokines (Figure 3c).

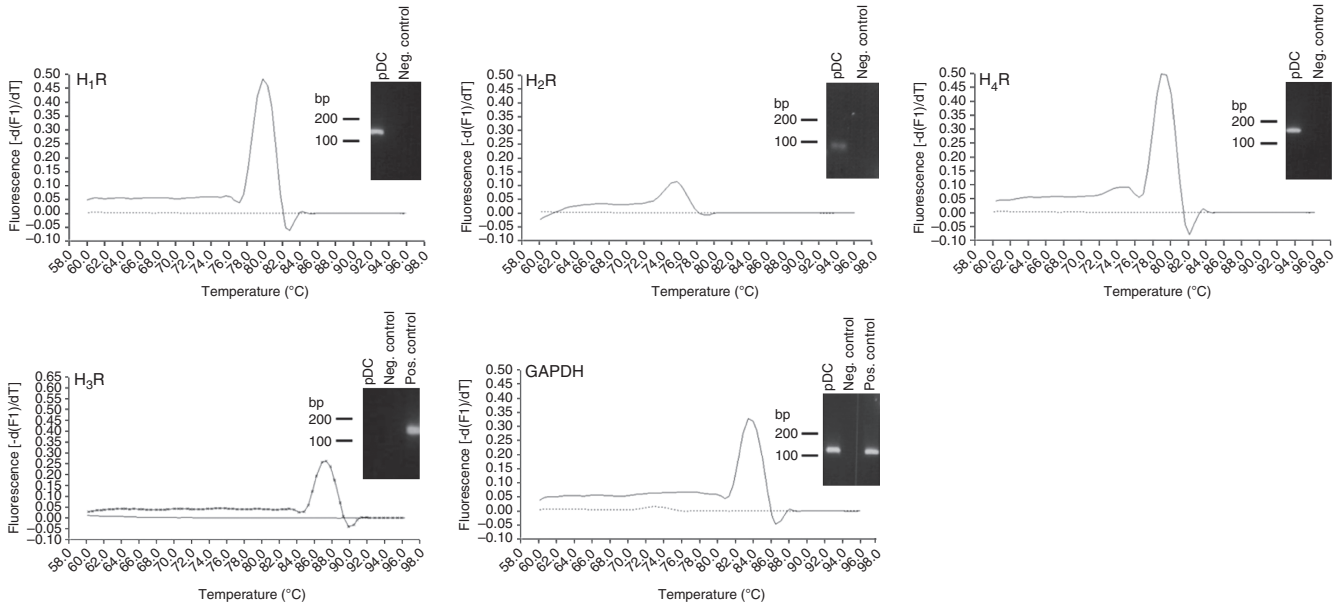

Figure 1. Plasmacytoid dendritic cells (pDC) isolated from buffy coats express the histamine receptors H_1R, H_2R, and H_4R at the mRNA level. Representative real-time PCR melting peaks and agarose gel bands of the PCR products of two independent experiments are depicted (continuous line, pDC; dotted line, negative control without reverse transcription; line with boxes, H_3R-transfected HEK cells as positive control for H_3R amplification). GAPDH, glyceraldehyde-3-phosphate dehydrogenase; H_xR, histamine receptor.

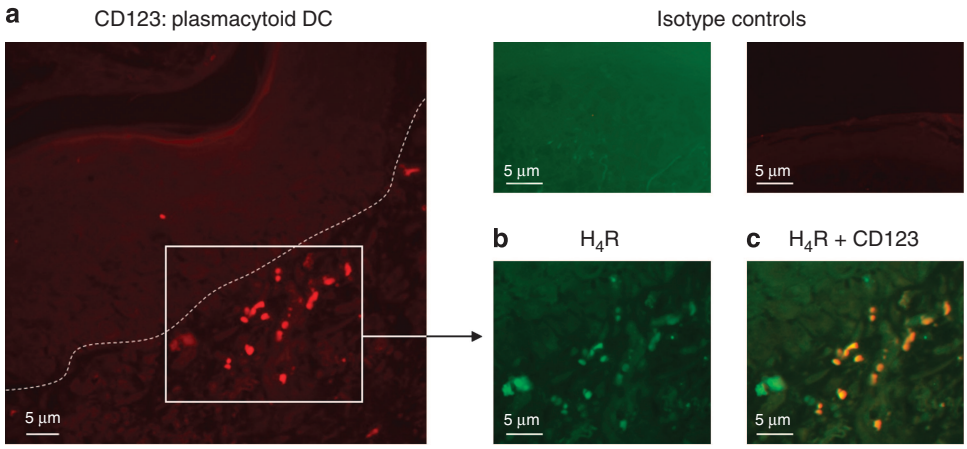

Figure 2. The H_4R is expressed *in situ* on plasmacytoid dendritic cells in psoriasis skin. Immunofluorescence staining of skin from psoriasis patients with anti-CD123 (**a**) and anti-H_4R (**b**) shows colocalization of the H_4R on CD123 positive cells in the dermis (**c**). One representative experiment of four is shown. The location of the basement membrane is indicated by the white scattered line. Bar = 5 μm. H_xR, histamine receptor.

Histamine mediates the downregulation of CpG-induced TNF-α, IFN-α, and CXCL8 secretion, in particular, in pDC derived from psoriasis patients

It has been described previously that pDCs are the principal producers of IFN-α and that they also secrete high amounts of TNF-α and of the chemokines CXCL8 and CXCL10 (Ito *et al.*, 2004). We confirmed this finding in our study and additionally show that stimulation of pDC with the H_4R-specific agonist 4-methylhistamine (4-MH) downregulated the secretion of TNF-α, IFN-α, and CXCL8 into the cell culture supernatant. In presence of the selective H_4R antagonist JNJ7777120, the H_4R agonist-induced downregulation of cytokines was completely blocked, whereas the histamine effect was only blocked partially (Figure 4a). As pDC also express the H_1R and H_2R on mRNA level (Figure 1), we tested in addition agonists at these receptors. Cytokine secretion was not influenced by stimulation with the H_1R agonist 2-pyridylethylamine, but we observed downregulation after stimulation with the H_2R agonist amthamine (Figure 4b). For TNF-α and IFN-α, the involvement of the H_2R was already shown previously by experiments using H_2R antagonists (Mazzoni *et al.*, 2003), for CXCL8 this finding is new. With none of the used histamine receptor agonists, we did observe significant differences in the secretion of the chemokine CXCL10 (Figure 4a and b).

Interestingly, in pDC derived from psoriasis patients (expressing higher levels of the H_4R as shown in Figure 3) histamine-induced downregulation of cytokines was more pronounced than in healthy controls (Figure 5). For IFN-α, we observed a downregulation by 25% in healthy controls and 45% in psoriasis patients, for TNF-α 30% and 85% and for CXCL8 15% and 75%, respectively. In psoriasis patients even the secretion of CXCL10 was reduced by 45%, in contrast in healthy controls no effect on CXCL10 was observed at all (Figures 4 and 5).

H_4R stimulation induced migration of pDC

The effect of H_4R stimulation on the migration of pDC was assessed by F-actin polymerization as readout for cytoskeleton reorganization and by cell migration assays. The H_4R agonist 4-MH and C3a as positive control (Gutzmer *et al.*, 2006) induced F-actin polymerization after short periods of stimulation and pre-incubation with the H_4R antagonist JNJ7777120 blocked H_4R agonist induced F-actin polymerization (Figure 6a and b). In accordance with these findings, we observed higher migration of pDC in response to the H_4R agonist and C3a in comparison to non-stimulated cells (Figure 6c and d).

DISCUSSION

An increased number of mast cells is present in the upper dermis and even in the epidermis of psoriasis patients (Cox, 1977; Harvima *et al.*, 2008) and the appearance of degranulated mast cells is an early feature of developing psoriasis lesions (Schubert and Christophers, 1985). Furthermore, the concentration of the most important mast cell mediator histamine is increased in psoriasis lesions (Petersen *et al.*, 1998). Also pDCs are found early in lesional skin of psoriasis patients (Wollenberg *et al.*, 2002) and have an important role in the pathology of this inflammatory skin disease (Nielsen, 1991; Nestle *et al.*, 2005). These findings show that pDC and histamine come into contact in lesions of psoriasis; nevertheless, the functional role of histamine on pDC is not elucidated in detail yet. One previous study was performed showing histamine-induced downregulation of IFN-α and TNF-α secretion from pDC, an effect that was mainly attributed to the H_2R (Mazzoni *et al.*, 2003). However, as the most recently discovered H_4R (Nakamura *et al.*, 2000) was not considered in this study, we aimed to elucidate the expression, regulation, and functional role of the H_4R on pDC.

In the present study, we show for the first time that pDC, isolated from peripheral blood and pDC *in situ* in psoriasis lesions, express the H_4R. When measuring H_4R expression in different patient groups we observed increased expression of the H_4R on pDC derived from patients with psoriasis, whereas for atopic dermatitis no significant difference was observed. Interestingly, the high level of H_4R expression on

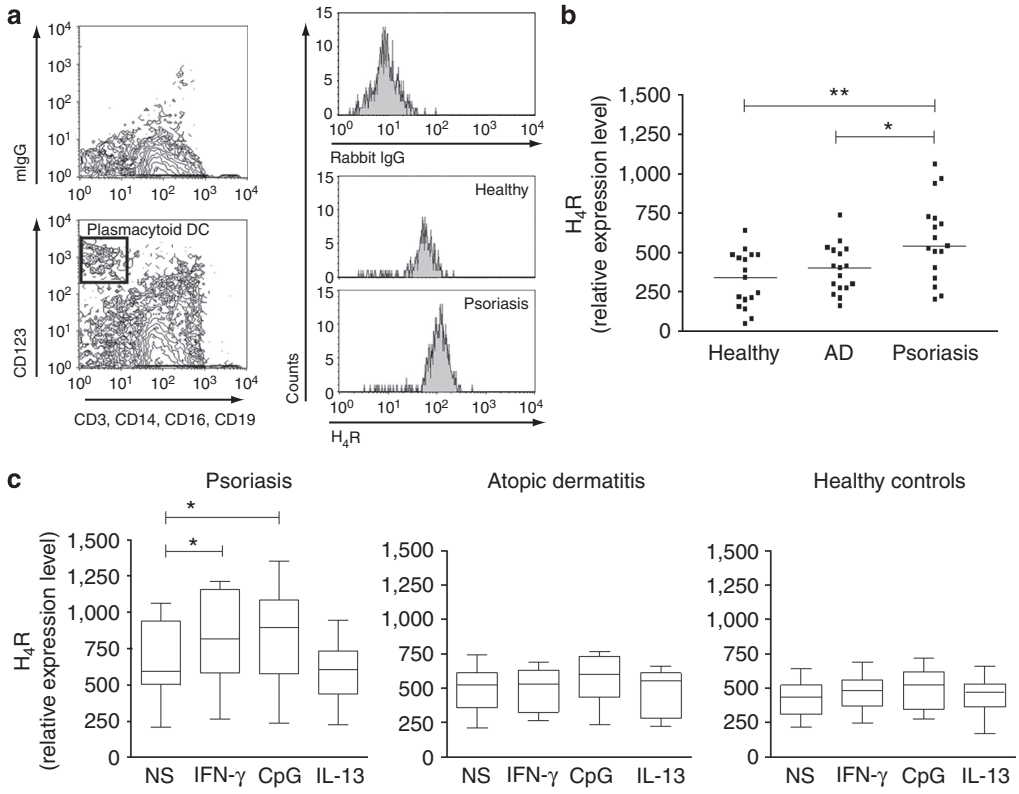

Figure 3. The H_4R is highly expressed on blood plasmacytoid dendritic cells (pDC) derived from psoriasis and upregulated by IFN-γ and CpG. (**a**) pDC in peripheral blood mononuclear cells (PBMC) were identified by cell surface staining with CD123 and negative lineage staining (the percentage of pDC in healthy controls was ~1%, in atopic dermatitis (AD) 1.1%, and in psoriasis 0.6% of whole PBMC). Representative histograms of isotype staining and H_4R staining on healthy and psoriasis pDC are shown. Panel **b** depicts a summary of all performed experiments (individual values and median of 17 independent experiments per group are shown). (**c**) PBMCs were stimulated for 48 hours with 20 ng ml^{-1} IFN-γ, 10 μg ml^{-1} CpG, or 50 ng ml^{-1} IL-13 and H_4R expression on pDC was determined using flow cytometry. Box plots of seven independent experiments performed with pDC derived from patients with psoriasis, atopic dermatitis, and healthy controls are shown (the relative expression levels of H_4R were calculated as follows: specific staining times 100 divided by isotype staining). H_xR, histamine receptor. *$P<0.05$, **$P<0.005$.

psoriasis pDC did further increase on stimulation with IFN-γ and CpG. IFN-γ is a predominant cytokine found in lesions and blood of psoriasis patients (Szabo *et al.*, 1998; Arican *et al.*, 2005), where it can interact with pDC and upregulate H_4R expression. The regulation of the H_4R on IFN-γ stimulation was also observed on other DC subtypes involved in inflammatory skin diseases, such as inflammatory dendritic epidermal cells (Dijkstra *et al.*, 2008) and slanDC (Gschwandtner *et al.*, 2011). In the present study, we also found upregulation of the H_4R on stimulation with CpG, which is a potent activator of pDC in general. In contrast, the Th2-associated cytokine IL-13 did not have an effect on H_4R expression on psoriasis pDC. Interestingly, we did not observe regulation of the H_4R by the tested cytokines in pDC derived from healthy controls or patients with atopic dermatitis. Together with the *per se* higher H_4R expression on pDC from psoriasis patients, these findings are the first showing disease-dependent regulation of histamine receptor expression in psoriasis. The mechanisms underlying this differential regulation of H_4R expression in psoriasis still need to be elucidated and will be subject to future studies. Of note, recently the regulation of the H_4R expression on the genetic level was reported, e.g., polymorphisms in the *HRH4* gene were shown to be associated with high H_4R expression in atopic dermatitis (Yu *et al.*, 2010b) and the *HRH4* gene copy number was significantly increased in patients with lupus erythematosus (Yu *et al.*, 2010a).

As our data shows that pDC in the blood as well as in the inflamed skin express high levels of the H_4R and are thereby able to respond to histamine, which is released by basophils in the circulation (Bochner, 2000) and by mast cells in the dermis of psoriasis patients (Krogstad *et al.*, 1997), we continued to identify the functional relevance of the H_4R on pDC. Therefore, we investigated the secretion of the most important pDC-derived cytokines and chemokines: IFN-α, TNF-α, CXCL10 (IP-10), and CXCL8 (IL-8) (Hochrein *et al.*, 2002). After stimulation with histamine, binding to all four histamine receptors or a H_4R-specific agonist, we observed decreased CpG-induced secretion of TNF-α, IFN-α, and CXCL8, whereas for the chemokine CXCL10 no effect was detected. By experiments using the H_4R selective antagonist JNJ7777120, we showed that the H_4R agonist-induced downregulation of cytokines could be completely blocked. In contrast, the histamine-induced downregulation was only partially blocked, indicating that also another histamine receptor is involved. Indeed, a previous study showed H_2R-mediated downregulation of IFN-α and TNF-α secretion from pDC (Mazzoni *et al.*, 2003). In their study, Mazzoni *et al.*

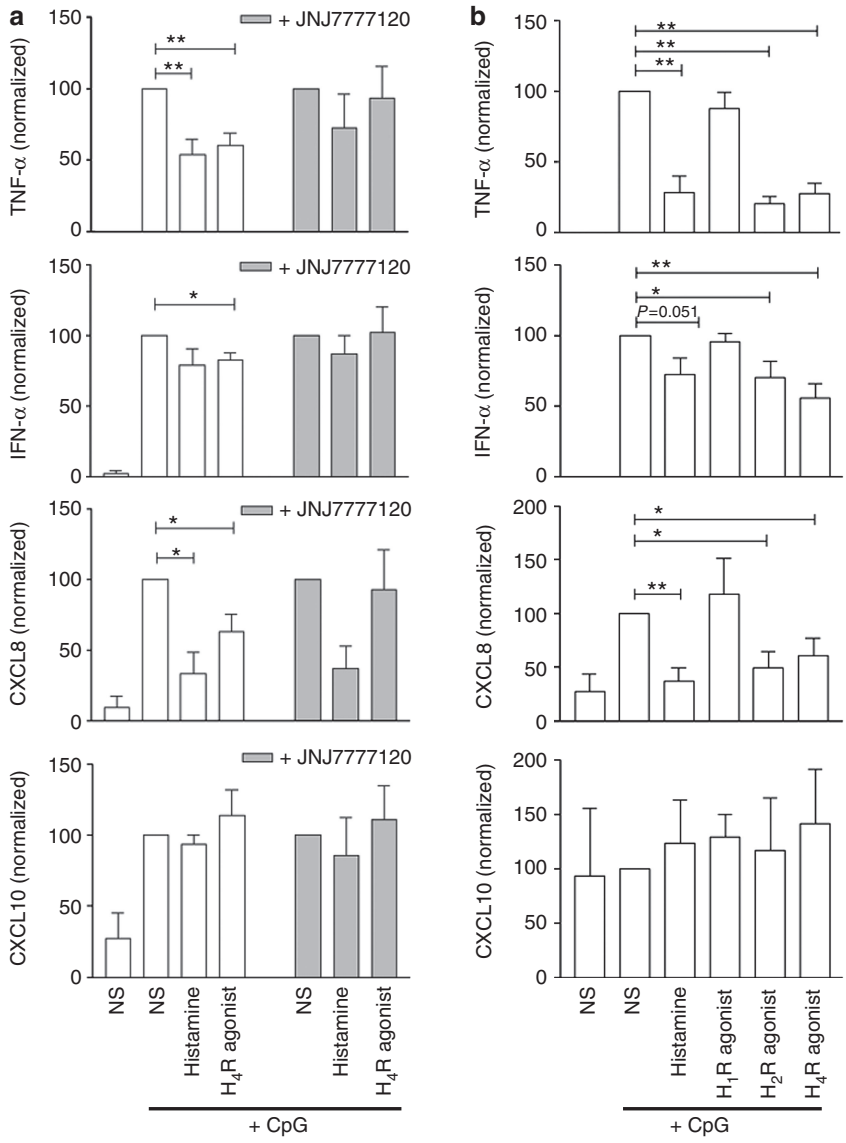

Figure 4. Histamine decreases tumor necrosis factor (TNF)-α, IFN-α, and CXCL8 secretion from plasmacyloid dendritic cells (pDC). pDCs were isolated from buffy coats and activated for 48 hours with IL-3. (**a**) pDCs were stimulated with 10 μM histamine or H_4R agonist 4-methylhistamine (4-MH) in combination with 10 μg ml^{-1} CpG. In blocking experiments, the cells were incubated with the H_4R antagonist JNJ7777120 30 minutes before histamine receptor stimulation. After 24 hours, the cytokine content in supernatants was determined by ELISA (mean and SEM of 8, 7, 5, and 7 independent experiments are shown). (**b**) The H_1R agonist 2-pyridylethylamine did not reduce CpG-induced cytokine production. The H_2R agonist amthamine downregulated TNF-α, IFN-α, and CXCL8 production to a similar extent as the H_4R agonist 4-MH. CXCL10 was not affected by histamine receptor stimulation (mean and SEM of seven independent experiments are shown). The mean levels of cytokines produced on CpG stimulation were as follows: 1,858 pg ml^{-1} of TNF-α, 3,608 pg ml^{-1} of IFN-α, 724 pg ml^{-1} of CXCL8, and 1,077 pg ml^{-1} of CXCL10. NS, non-stimulated. *$P<0.05$, **$P<0.005$.

described histamine-induced downregulation of IFN-α with varying magnitude depending on the TLR ligand used for stimulation; however, the effect was more pronounced than in our study. For TNF-α, the downregulation observed after histamine stimulation was comparable to the experiments performed by us. In their study, Mazzoni *et al.* showed only partial blockade of histamine using the H_2R selective antagonist ranitidine and the tested H_1R and H_3R antagonists were ineffective in blocking histamine-induced downregulation of cytokine production. In the present study, we used specific histamine receptor agonists to confirm, that the H_1R is not involved in the regulation of cytokine secretion from pDC and to show that cytokine production is downregulated to a similar extent by H_2R and H_4R agonists. These findings indicate that both receptors have a central role in downregulating the proinflammatory cytokine production in pDC. The combined action of the H_2R and the H_4R on cytokine secretion was also observed previously in monocyte-derived DC (Gutzmer *et al.*, 2005).

The cytokines downregulated by histamine stimulation on pDC—TNF-α, IFN-α and CXCL8—have a major role in the pathology of psoriasis as they lead to the activation and

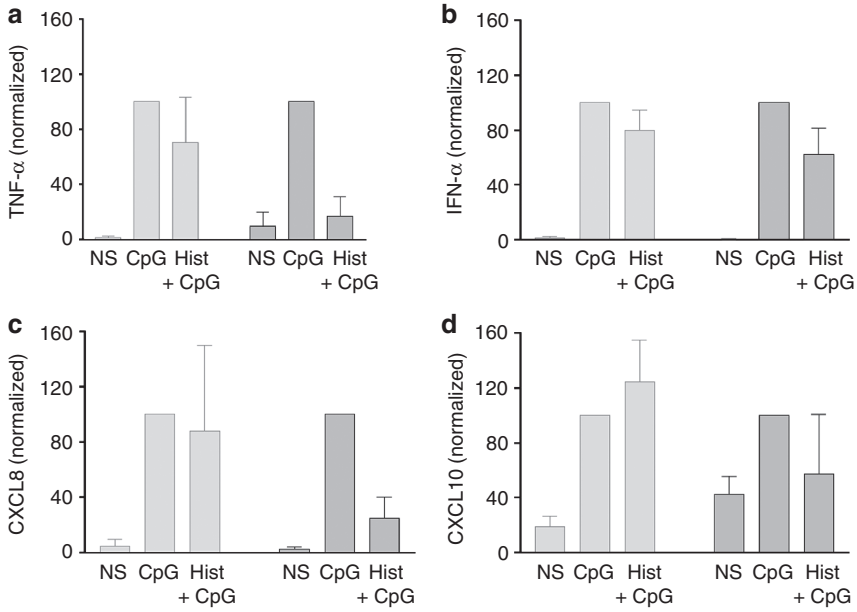

Figure 5. The downregulation of cytokine production by histamine is more pronounced in plasmacytoid dendritic cells (pDC) derived from psoriasis patients. pDC isolated from the peripheral blood of healthy controls (light gray bars) or patients with psoriasis (dark gray bars) were activated for 48 hours with IL-3 and were thereafter stimulated with 10 μM histamine in combination with 10 μg ml^{-1} CpG. After 24 hours of stimulation, the cytokine content in supernatants was determined by ELISA. Mean and SEM of four independent experiments are shown and the mean levels of cytokines produced were comparable to the ones given in Figure 4. NS, non-stimulated.

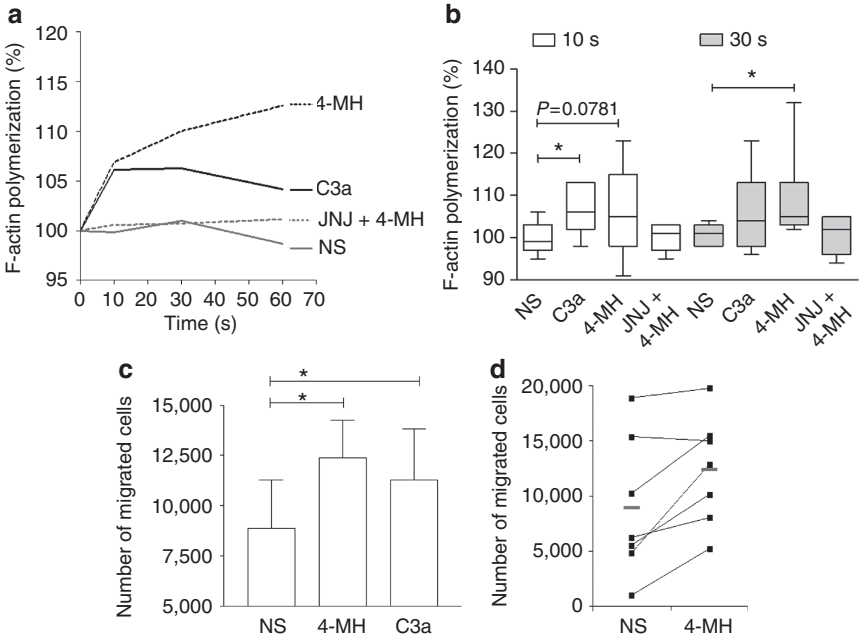

Figure 6. H_4R stimulation induces F-actin polymerization and migration of plasmacytoid dendritic cells (pDC). (**a**, **b**) Isolated pDCs were activated for 48 hours with IL-3. Subsequently pDC were left untreated or stimulated with 10 μM of the H_4R agonist 4-methylhistamine (4-MH) or 1 μg ml^{-1} C3a as positive control. In the blocking experiments, the cells were incubated with JNJ7777120 (JNJ) 15 minutes before stimulation with 4-MH. (**a**) Depicts the time course of F-actin polymerization (mean of seven independent experiments) and in (**b**) the statistical analysis of the time points 10 and 30 seconds is depicted (box plot of seven independent experiments). (**c**, **d**) For the migration experiments, phosphate-buffered saline as control or 10 μM 4-MH or 1 μg ml^{-1} C3a was added to the bottom chambers. The chambers were covered with a 5 μm membrane and on top of the membrane calcein-labeled pDC suspension was added. After 90 minutes, the amount of cells that migrated to the bottom chamber was analyzed fluorometrically. A box plot of seven independent experiments (**c**) and the individual experiments for 4-MH stimulation (**d**) are shown. H_xR, histamine receptor; NS, non-stimulated. *$P<0.05$.

infiltration of T cells, affect the Th1/Th2 balance in their microenvironment and activate keratinocytes to produce proinflammatory cytokines (Nestle *et al.*, 2005; Fantuzzi *et al.*, 2008). Because of these widespread functions the histamine-mediated downregulation of pDC-derived cytokines influences the local cellular immune response in

many ways. The important role of histamine in psoriasis is supported by the finding that in pDC derived from patients with psoriasis the downregulation of cytokines was more pronounced than in pDC derived from healthy controls. In psoriasis patients, we even detected downregulation of the chemokine CXCL10, an effect that was not observed in healthy controls. These findings indicate that pDC in psoriasis do not only show increased expression of the H_4R, but that they also demonstrate a different functional response to stimulation with histamine.

Another important function of pDC is migration, leading to their initial recruitment to the inflamed skin and on their activation and maturation to the mobilization to lymph nodes. Although several mediators have been shown to be involved in pDC migration, such as C3a and C5a (Gutzmer *et al.*, 2006), CXCR4 (Pablos *et al.*, 1999), and ChemR23 (Vermi *et al.*, 2005; Albanesi *et al.*, 2009), the process of pDC migration is not fully elucidated yet. In the present study, we identify histamine as another chemotactic stimulus for pDC. We observed F-actin polymerization and the active migration of pDC in response to H_4R stimulation. As histamine is an early product in lesions of psoriasis it might be one of the initial signals leading to the immigration of pDC. A recent study investigating the migration of pDC to lymph nodes reported a crucial role of histamine in pDC migration to the lymph node following peptidoglycan injection (Dawicki *et al.*, 2010). This dependence seemed to be specific for pDC, as the migration of CD11b DC did not rely on histamine. The study showed that the H_2R antagonist ranitidine was only partially effective in blocking the migration, whereas H_1R antagonists were ineffective. H_4R antagonists were not investigated in this study, but because of our findings one could assume that the H_4R also has a role in the migration to lymph nodes.

Taken together, our study provides good evidence that not only, as previously described, the H_2R but also the H_4R influences pDC migration and cytokine production. Together with other studies describing anti-inflammatory properties of the H_4R on different antigen-presenting cell subsets (Gutzmer *et al.*, 2005; Dijkstra *et al.*, 2008; Gschwandtner *et al.*, 2010), these findings indicate that histamine has an important role in downregulating immune responses in the skin. H_4R ligands, alone or in combination with H_2R ligands, might therefore be of potential in the treatment of inflammatory skin diseases. This might be true especially for psoriasis, as we could demonstrate significantly higher basal H_4R expression, differential receptor regulation by cytokines and a more pronounced response to stimulation of the H_4R in psoriasis.

MATERIALS AND METHODS

Isolation and culture of PBMC and pDC

Peripheral blood samples were taken from patients with severe extrinsic atopic dermatitis or psoriasis, patients without inflammatory skin disease served as controls. Patients were diagnosed and treated in our department; they did not receive systemic treatment during a 2 weeks period before blood withdrawal. All participants gave their written informed consent. PBMC were separated by density centrifugation on lymphoprep (Fresenius Kabi Norge AS, Oslo, Norway) and erythrocytes were removed by incubation with Gey's lysis buffer. For the isolation of pDC, buffy coats from anonymous healthy donors were obtained from the local blood bank. pDCs were isolated by negative magnetic selection using pDC isolation kit from Miltenyi Biotech (Bergisch-Gladbach, Germany). The isolated pDC had a purity of at least 90% based on expression of CD123, BDCA-2, and HLA-DR, and lack of expression of CD3, CD14, CD16, and CD19.

PBMC and pDC were cultured in Iscoves medium supplemented with 2 mM L-glutamin, 100 $\mu g\,ml^{-1}$ penicillin/streptomycin, 1 × non-essential amino acids, 0.05 $mg\,ml^{-1}$ gentamycin, and 6% v/v fetal calf serum at 37 °C in a humidified atmosphere containing 5% CO2. pDCs were stimulated with IL-3 (10 $ng\,ml^{-1}$, Immunotools, Friesoythe, Germany) to avoid apoptosis and to acquire an immature pDC phenotype with upregulation of co-stimulatory molecules (Grouard *et al.*, 1997) for 48 hours before starting of experiments.

Messenger RNA isolation, reverse transcription, and quantitative real-time PCR

Isolated pDCs were washed in phosphate-buffered saline (PBS) and lysed for RNA isolation using the Mini RNA Isolation II kit (Zymo Research, Orange, CA) and reverse transcription was performed with the Quantitect reverse transcription kit (Qiagen, Hilden, Germany). As control, complementary DNA of H3R-transfected HEK cells was prepared analogously. Real-time quantitative PCR was performed on a LightCycler (Roche Molecular Biochemicals, Mannheim, Germany) using SYBR Green with Quantitect primer assays for H_1R (QT00199857), H_2R (QT00210378), H_3R (QT00210861), H_4R (QT00032326), and glyceraldehyde-3-phosphate dehydrogenase (QT01192646) as housekeeping gene according to the manufacturer's instructions (Qiagen). The following PCR settings were used: an initial activation step of 15 minutes at 95 °C with ramp 20 °C per second was followed by three-step cycling (45 cycles): denaturation 15 seconds, 94 °C; annealing 20 seconds, 55 °C; extension 20 seconds, 72 °C (all three with ramp 2 °C per second). Melting curve analysis was performed from 60 to 90 °C with ramp 20 °C per second. The amount of target genes relative to the reference glyceraldehyde-3-phosphate dehydrogenase and the melting point was analyzed using Relative Quantification software (Roche Molecular Biochemicals). To visualize the amplification products agrose gel electrophoresis was performed with 2% agarose (Roth, Karlsruhe, Germany) in 1 × Tris-Borat-EDTA buffer (Roth).

Immunofluorescence staining of the H_4R and pDC

Paraffin embedded sections (5 μm) from skin biopsies derived from patients diagnosed with psoriasis were transferred to SuperFrost Plus microscope slides (Menzel-Gläser, Braunschweig, Germany). After drying, the sections were deparaffinated and treated with antigen unmasking solution (Vector Laboratories, Burlinghame, CA). After washing, the sections were incubated in goat serum for 1 hour at room temperature and then stained with anti-H_4R (SantaCruz Biotechnology, Santa Cruz, CA) or rabbit IgG overnight. The next day the staining was continued with secondary antibody (biotinylated goat anti-rabbit antibody, Vectastain ABC kit, alkaline phosphatase rabbit; Vector Laboratories) for 1 hour at room temperature and avidin-fluorescein (Vector Laboratories) for 5 minutes at room temperature. Following this, the slides were stained with anti-CD123 (BD Pharmingen, Heidelberg, Germany) overnight

at 4 °C. The stained sections were covered with mounting medium (Vectashield; Vector Laboratories) and coverslips. Fluorescence images were obtained with a Zeiss Axiolab microscope with AxioCam MRm, and images were acquired with the program AxioVS40 (version 4.6.1.0; Carl Zeiss MicroImaging GmbH, Göttingen, Germany).

Flow cytometric analysis of H_4R expression

The cells were washed twice in PBS and then incubated for 20 minutes in PBS-containing human IgG (FcγR-blocking buffer). The cell surface was stained with CD123-PECy5 (BD Pharmingen) and lineage markers (CD3-PE, CD14-PE, CD16-PE, and D19-PE) or the respective isotype controls. Subsequently cells were fixed and permeabilized (Fixation/Permeabilization Kit, eBioscience, San Diego, CA). Intracellular staining was performed with H_4R antibody recognizing amino acids 194–303 (SantaCruz Biotechnology) or polyclonal rabbit isotype control (R&D Systems, Wiesbaden, Germany), followed by labeling with goat anti-rabbit-FITC (Beckman Coulter, Krefeld, Germany). CD123, linage, and H_4R positivity of the cells were assessed by flow cytometry (FACS Calibur; Becton Dickinson, Heidelberg, Germany).

For the measurement of H_4R expression in response to cytokine stimulation the cells were incubated for 48 hours with 20 ng ml^{-1} IFN-γ (R&D Systems), 50 ng ml^{-1} IL-13 (Peprotech, Hamburg, Germany) or 10 μg ml^{-1} CpG oligonucleotide ODN2216 (5′-ggGGG ACGATCGTCgggggG-3′, TIB MOLBIOL, Berlin, Germany).

ELISA

1×10^5 pDCs per well were cultured in IL-3-containing medium (10 ng ml^{-1}) for 48 hours and were either not stimulated or stimulated with 10 μM histamine (Alk-Scherax, Wedel, Germany), the H_1R agonist 2-pyridylethylamine, the H_2R agonist amthamine or the H_4R agonist 4-MH (all from Tocris Bioscience, Bristol, UK) and activated with 10 μg ml^{-1} CpG ODN2216 (TIB MOLBIOL) for 24 hours. For blocking experiments the cells were treated with the H_4R antagonist JNJ7777120 (Sigma-Aldrich, Deisenhofen, Germany) 30 minutes before stimulation with receptor agonists. Cell-free supernatants were collected after 24 hours and the cytokine content was analyzed using commercially available ELISA according to the manufacturer's instructions: IFN-α (IBL, Hamburg, Germany), TNF-α (eBioscience), CXCL8, and CXCL10 (R&D Systems).

Assessment of F-actin polymerization

Nitrobenzoxadiazole-phallacidin (Invitrogen, Darmstadt, Germany) staining of pDC was carried out as described previously (Gutzmer *et al.*, 2006). Briefly, cells were resuspended at a concentration of 1×10^6 cells per ml in PBS lacking Ca^{2+} and were stimulated with the H_4R agonist 4-MH (10 μM, Tocris Bioscience) or C3a as positive control (1 μg ml^{-1}, Calbiochem, Darmstadt, Germany) for short periods of time (0, 15, 30, and 60 seconds) at room temperature. For blocking experiments cells were incubated with the H_4R antagonist JNJ7777120 (Sigma-Aldrich) 15 minutes before addition of 4-MH. Following stimulation, cells were fixed using 3.7% formaldehyde for 60 minutes. Lysophosphatidylcholine (20 μg ml^{-1}, Sigma-Aldrich) and 3.7×10^{-7} M nitrobenzoxadiazole-phallacidin were added to the sample and incubated for a period of 60 minutes in the dark. Nitrobenzoxadiazole-phallacidin-stained cells were analyzed on a Becton Dickinson FACScalibur with a linear fluorescence channel (FL-1), in which the fluorescence is proportional to F-actin content. Relative F-actin content was normalized to the mean channel fluorescence time as zero.

Chemotaxis assay

The chemotactic activity of pDC was determined using the micro chamber technique ChemoTx System (Neuro Probe, Gaithersburg, MD). Isolated pDC precursors were cultured for 48 hours in medium containing IL-3 (10 ng ml^{-1}). Then, 1×10^6 pDC per ml were labeled in PBS with 5 μg ml^{-1} calcein-AM (Molecular Probes, Leiden, the Netherlands) for 30 minutes at 37 °C. A volume of 27 μl of the chemotactic stimulus or control medium (PBS) was filled in the bottom chamber of disposable 96-well plates (Neuro Probe) and covered with a polycarbonate filter with a pore size of 5 μm. 1×10^5 labeled pDCs were loaded in a volume of 25 μl PBS on top of the membrane. After incubation for 90 minutes at 37 °C, remaining cells on top of the membranes were carefully removed by wiping. Calcein fluorescence in the bottom chamber was measured using the FLUOstar Galaxy (BMG Labtechnologies, Offenburg, Germany). The number of migrated cells equivalent to calcein fluorescence was calculated from a standard curve with known cell number.

Statistical analysis

Before statistical analysis a normality test was performed, for calculation of *P*-values either an unpaired *t*-test (Figure 1), Wilcoxon matched pairs test (Figures 3 and 6b), or paired *t*-test (Figures 4 and 6c) was used; a *P*-value below 0.05 was regarded as significant. $^*P<0.05$ and $^{**}P<0.005$. The program GraphPad Prism version 5 (GraphPad Software, San Diego, CA) was used for statistical analysis.

Ethics

The investigation of the role of histamine receptors in inflammatory skin diseases was approved by the local ethics committee of the Hannover Medical School (Vote No. 4253) and was conducted according to the Declaration of Helsinki Principles.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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