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Histamine regulates multiple functional activities of murine and monocyte-derived human dendritic cells via different receptors

by Tünde Simon

Supervisor: Éva Rajnavölgyi

UNIVERSITIY OF DEBRECEN
DOCTORAL SCHOOL OF MOLECULAR CELL AND IMMUNE BIOLOGY

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By Tünde Simon, molecular biologist

Supervisor: Prof. Dr. Éva Rajnavölgyi

Doctoral School of Molecular Cell and Immune Biology, University of Debrecen

Head of the **Examination Committee**:	Prof. Dr. Margit Zeher
Members of the Examination Committee:	Dr. Andrea Szegedi
	Dr. Attila Mócsai

The Examination takes place at the 3rd Department of Internal Medicine, University of Debrecen
Debrecen, 2012.06.04. at 11 a.m.

Head of the **Defense Committee**:	Prof. Dr. Margit Zeher
Reviewers:	Prof. Dr. László Mátyus
	Prof. Dr. Jerzy Jochem
Members of the Defense Committee:	Dr. Andrea Szegedi
	Dr. Attila Mócsai

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Introduction

The mammalian organism is protected against invading microorganisms at the interfaces of the external environment (skin, respiratory tracts, gastrointestinal mucosa) by special sentinels, called dendritic cells (DCs).

1. Life cycle of DCs

DCs are generated in the bone marrow and they are seeded through the blood stream into peripheral lymphoid and non-lymphoid tissues, where they easily make contact with foreign particles or invading microbes. When continuously sampling their environment, at an immature stage of development, DCs capture different molecules by their cell surface receptors and internalize them via various mechanisms such as phagocytosis, macropinocytosis or receptor-mediated endocytosis. After processing of the antigenic sample it is fixed on the surface of DCs as peptides that are presented by major histocompatibility complex (MHC) molecules to antigen-specific T cells. Immature DCs are characterized by high capability of antigen capture and processing, but low T cell stimulatory capacity. Antigen uptake and inflammatory mediators promote DC maturation and migration to draining lymph nodes where they efficiently trigger an immune response by naive T cells. DC maturation is a complex and highly regulated process which include phenotypic and functional changes such as losing the ability of antigen capture, acquiring an increased capacity for T cell stimulation and upregulation of costimulatory molecules. Furthermore, maturation is accompanied by the modulation of the expression of some chemokine receptors. While CXCR1, CCR1, CCR5 expression is downregulated, the chemokine receptor CCR7 is induced together with DC maturation enabling the mobilization of DCs from the periphery to the lymph nodes. CCL19 (MIP-3ß) and CCL21, the ligands of CCR7 are produced constitutively in the T cell areas of lymph nodes. CCL21/6Ckine molecule expressed in lymphatic vessels also plays a role in directing these cells. Upon arrival to lymphoid compartments DCs are able to activate different subsets of lymphocytes by polarizing them to Th1, Th2, Th17 or Treg directions. This process is highly dependent on DC-derived signals that involve foreign peptide-MHC complexes expressed on the DC surface, co-stimulatory signals and cytokine/chemokine-mediated instructions. The interaction of the T cell receptor (TCR) with its specific ligand is indispensable as a first signal, whereas the second signals are mediated by B7-1 or B7-2 molecules (CD80, CD86) by triggering T cells through CD28 receptor and/or additional environmental stimuli. The cytokines produced by DCs and T cells

act in both directions and modulate the intensity and quality of the immune response (by the differentiation of Th1, Th2, Th17 and Treg cells). IL-12 or IFNγ produced by DCs can polarize T cells to Th1 direction, the IL-4 or IL-5 cytokines support Th2 polarization, whereas IL-10 induces immune suppression. The antigen presenting function of DCs is not restricted to the presentation of endogenous peptides by MHCI for $CD8^+$ T cells and exogenous peptides by MHCII molecules for $CD4^+$ T cells, as they also present lipids and glycolipids complexed with CD1 membrane proteins and endo- or exogenous antigens with MHCI molecules to elicit $CD8^+$ T cells, called cross-presentation.

Due to their multiple functions the proper instructions of DCs to other immune cells is essential for inducing immunological tolerance in the thymus and the periphery, or provoking an effective immune response. Depending on the type of the invading microorganism and the nature of stress factors or danger signals DCs can direct immune responses to various activities.

2. CD1a, a human DC marker and lipid presenting molecule

Members of the CD1 family of MHC-class-I-like glycoproteins, are phylogenetically conserved proteins, specialized to the presentation of hydrophobic ligands, such as self and foreign lipids and glycolipids. The CD1 proteins share sequence homology and overall domain structure with MHC class I molecules, being comprised of a heavy chain that is non-covalently associated with ß2-microglobulin. To our current knowledge five CD1 isoforms can be distinguished and based on their sequence can be further divided into three subgroups. Group1 is composed of CD1a, CD1b and CD1c molecules, group 2 and group 3 contain only one protein, CD1d and CD1e, respectively. Except from CD1e, all isoforms are cell surface proteins. Interestingly, the expression pattern of human CD1 proteins differs from other species, as in humans all five isoforms are present, while in mice only CD1d is expressed. Group 1 molecules preferentially present lipid antigens to „conventional" αß or innate γð T cells, while group 2 molecules (CD1d) present antigens to invariant NKT cells (iNKT). CD1a, a member of the group 1 family is expressed mainly on DCs. *In vivo* three different DC fractions can be isolated from human blood: $CD1a^+CD11c^+$, $CD1a^-CD11c^-$ and $CD1a^-CD11c^+$ populations. The double positive population is the direct precursor of LCs, and can be differentiated to LCs *in vitro*. CD1a expression is similar on resting and activated DCs.

3. Histamine and histamine receptors (HRs)

The effects of histamine are mediated by four HRs, all being typical heptahelical proteins the activation of which is mediated by G proteins. The first histamine receptor discovered (H_1R) possesses the lowest binding affinity to the amine and is linked mainly to

allergic reactions. It shares the typical features of G-protein-coupled receptors activating Gαq. The ligand binding to H_1R leads to increased cytosolic Ca^{2+} and cAMP levels and results in the activation of NFκB pathway. In the lung it mediates bronchoconstriction and increased vascular permeability. The H_1R is expressed in numerous cell types, including airway and vascular smooth muscle cells, hepatocytes, chondrocytes, nerve cells, endothelial cells and some immune cells.

Histamine receptor 2 (H_2R) is coupled to the adenylate cyclase and phosphoinositide second messenger systems by separate GTP-dependent mechanisms. Receptor binding also activates c-Fos, c-Jun and protein kinase C. The ligand binding affinity of H_2R exceeds that of the H_1R and similarly to H_1R, H_2R is also expressed in various cell types. Initially it was thought to regulate a limited number of activities such as heart contraction and gastric acid secretion however, it is now quite clear that various regulatory functions of histamine during cell proliferation, differentiation and immune response are exerted through H_2R activation.

Histamine receptor 3 (H_3R) is predominantly expressed in the central nervous system and is involved in the regulation of the adjustment of locomotor activity and circadian rhythm of the body.

In 2001 the existence of a fourth HR was identified. H_4R is typically expressed by cells of the immune system, preferentially peripheral hematopoietic cells including eosinophils, basophil granulocytes, mast cells, T and B lymphocytes and DCs. However, the presence of H_4R has recently been shown in tissues functionally distant from the immune system, like in the brain or liver. As H_4R is widely distributed in immune cells its role in inflammation, hematopoiesis and directing immune responses was suspected. The effects of H_4R are mediated through the activation of Gi/o proteins. As a result of a multistep signaling cascade Ca^{2+} flows into the cell, cAMP level is decreasing and the MAPK pathway is activated. The ligand binding affinity of H_4R is greater than that of H_1R or H_2R, but lower than that of the H_3R. The growing number of specific agonists and antagonists holds promise of novel tools in the therapy of inflammatory and/or autoimmune disorders.

4. Relationship of histamine and DCs

Observations proving that DCs express both HRs and their natural ligand histamine support the concept that DCs may be under the influence of histamine derived not only from professional histamine synthesizing cells, such as basophils and mast cells, but also from DCs themselves.

Differentiation of DCs is a complex and highly regulated process accompanied by phenotypical changes and the alteration in the expression of numerous molecules. Results

showed that both HDC protein expression and intracellular histamine content was increased during *in vitro* differentiation of DCs from human peripheral blood monocytes. In parallel with these the expression of functionally relevant cell surface molecules (CD80, CD86, CD40, CD45 and CD11c) was elevated, too.

Migration is an important and indispensable feature of DCs. Based on both *in vivo* and *in vitro* data, histamine is able to modulate DC migration via different HRs, since it induces intracellular Ca^{2+} mobilization and F-actin polymerization and beside these, histamine or HR agonists were found to be strong chemotaxins for resting/immature (iDCs), however they are ineffective for activated/mature (mDCs).

Increasing body of evidence supports that histamine has an impact on antigen presentation as well. Expression of costimulatory and accessory molecules (CD40, CD80, CD86) and MHC class II, involved in effective antigen presentation, are enhanced by histamine transiently on human monocyte-derived iDCs. Others showed that splenic DCs of $HDC^{-/-}$ mice kept under histamine-free conditions, displayed a more efficient *in vitro* antigen presentation as compared to cells from WT mice.

DCs are professional antigen presenting cells, which effectively prime naive antigen-specific T cells. DC maturation is associated with the synthesis of numerous cytokines and chemokines that act on T cell polarization. The most intensively studied cytokines include the Th1 type IL-12 and the Th2 or T regulatory type IL-10. The majority of experimental data support that histamine induces an altered cytokine expression in DCs, and favor Th2 polarization. Histamine inhibited LPS- or IFNγ-induced IL-12 responses and the production of some other pro-inflammatory cytokines such as IL-1ß and IL-6. In contrast, relevant Th2 cytokines and LPS-stimulated IL-8 and IL-10 synthesis were significantly increased in histamine-treated human monocyte-derived mDCs. Histamine was also shown to upregulate the production of Th2-attracting chemokines CCL17 and CCL22 and to downregulate IFNγ-induced CXCL10 production by human monocyte-derived iDCs.

Aims of the studies

During their life cycle DCs are often get exposed to histamine. This multifunctional amine is known to influence DC differentiation and is involved in the regulation of various DC functions. The activities of DCs may be associated with more than one HR, as shown by the role of H_1R and H_2R in the functional activities of these cells. However, after the discovery of the novel H_4R, some previous results await for re-evaluated. Thus, in our two experimental systems first we tested whether histamine had an effect on the functional activities of mouse DCs. The migration, adhesion, cytokine production and the antigen presenting function of DCs were investigated. Beyond these studies we also investigated the possible impact of the lastly discovered H_4R in mediating these histamine effects. A gene knock out (KO) mouse model and *in vitro* receptor blocking were used to answer these questions.

Specific questions addressed in the murine system:

- Comparison of HR expression in splenic and bone marrow-derived DCs
- Investigation of the effect of histamine on *in vitro* antigen presentation
- Testing the role of H_4R in histamine-modulated antigen presentation in WT and $H_4R^{-/-}$ DCs
- Testing the role of histamine and H_4R in DC migration and adhesion
- The role of H_4R in the cytokine production of *in vitro* activated DCs
- Comparison of cytokine production of DCs isolated from WT and $H_4R^{-/-}$ mice following *in vivo* activation

It is also known that $CD1a^-$ and $CD1a^+$ DCs derived from human monocytes exhibit distinct functional characteristics being the $CD1a^+$ subset more inflammatory as compared to its $CD1a^-$ counterpart. The cytokine, chemokine production and phagocytic function of the two subpopulations are also different. We postulated that histamine could affect (perhaps differently) the activities of the two subsets and the results would enable us in better understanding the mechanism of histamine on DCs and further the diseases in which these cells were involved. Therefore, in the second set of experiments we tested how histamine influences the development and functional activities of $CD1a^-$ and $CD1a^+$ DC subsets

differentiated *in vitro* from human $CD14^+$ blood monocytes. We also tested the HR dependence of the identified effects.

Specific questions addressed in the human system:

- Comparison of HR expression in the functionally distinct $CD1a^-$ and $CD1a^+$ DC subpopulations derived from human blood monocytes
- Testing the effect of histamine on the differentiation of DC subsets
- Investigation of the modulatory role of histamine on $CD1a^-$ and $CD1a^+$ DC activation
- Impact of histamine on DC cytokine and chemokine production of DCs
- Testing the effect of histamine on *in vitro* DC migration by using specific receptor blockers

Methods

Mouse studies

1. Animals

In the experiments wild-type (WT) and $H_4R^{-/-}$ (H_4R-knockout, H_4R-KO) mice with Balb/c background were used at 2–3 month of age.

2. Primary cells

a. DC isolation

DCs were purified from spleens of WT and $H_4R^{-/-}$ mice by CD11c-MACS immunomagnetic bead selection. The homogeneity of the cell population was controlled by flow cytometry.

b. In vitro differentiated mouse DCs

In some experiments *in vitro* differentiated DCs generated according to procedure of Lutz *et al.* were applied. The ratio of $CD11c^+$ DCs was controlled by flow cytometry.

3. Phenotypic characterization of DCs

The expression of HRs (H_1R, H_2R, H_4R) and relevant DC markers (CD11c, CD11b, MHCII, CD40, CD80, CD86) was performed by flow cytometry. Part of the cells was treated with histamine (1 µM; 10 µM). The length of histamine incubation of splenic DCs was 4 or 19 hours, whereas bone marrow-derived DCs were treated on day 13 with histamine for 24 hours. 1 µg/ml lipopolysaccharide (LPS) was used to activate DCs (24 hours, 37ºC).

4. *In vitro* antigen presentation and IL-2 production

The antigen presenting capacity of WT and $H_4R^{-/-}$ DCs was measured with an *in vitro* assay. Freshly isolated mouse splenic DCs ($2x10^3$ cells/well) were co-cultured with the 5/4E8 T cell hybridoma cell line ($2x10^4$ cells/well), specific for the human aggrecan G peptide (24 hours, 37ºC). Wells contained 200 µl culture medium and 1 µg/ml human aggrecan G peptide (ATEGRVRVNSAYQDK). 24 hours later IL-2, produced by the activated 5/4E8 hybridoma cells, was measured by sandwich ELISA according to the manufacturer's instructions.

DCs were challenged with different concentrations (0.1 µM-100 µM) of histamine, or 4-methylhistamine (H_4R agonist, 4-MH, 1-100 µM) administered together with the aggrecan peptide. In some experiments HR antagonists famotidine (H_2R, 10 µM) and JNJ10191584 (H_4R, 10 µM) were applied 1 hour before histamine (1 µM) treatment.

5. *In vitro* migration assay

Migratory response of WT and $H_4R^{-/-}$ DCs was examined with a Transwell migration assay. DCs were suspended in 10^6 cells/ml then after the preincubation of the Transwell system the upper chamber with the membrane was filled with 200 µl of cell suspension ($1x10^6$ cells/ml)

in RPMI-1640 and 600 µl of medium with or without histamine (1, 10, 100 µM) or 4-MH (1, 10, 50 µM) was added to the lower chambers. Migration was allowed for 2 hours (37 ºC). The number of transmigrated cells in the bottom chamber was counted by flow cytometry normalized to polystyrene microparticles.

In the experiments using differentiated DCs 1 µM histamine was added to the cells on days 0, 3, 6 and 8 of the differentiation process. Migration of 10-day bone marrow-derived DCs of WT and $H_4R^{-/-}$ was compared by using the same experimental protocol.

6. Cell adhesion assay

Adhesion characteristics of DCs were determined with a real-time impedance-based method by using the xCELLigence RTCA SP system. Prior to the experiments the bottom of 96 well plates (E-plate®96) containing electrodes were coated with 25 µl 0.025% human plasma fibronectin containing 0.1% gelatin solution for 20 min at 4 °C. After the removal of coating 100 µl complete medium (RPMI-1640) was added as a reference substance of baseline measurements, then DCs were seeded into wells (50.000 cells/well/100 µl) in triplicates. Values of impedance (Z) were recorded in a real time mode at 10 kHz for 12 hours. Impedance was represented by the cell index (CI) values ($(Z_n-Z_0)/F_i$ [Ohm]/15[Ohm]; Z_0: background resistance, Z_n: individual time point resistance, F_i: constant characteristic for frequency) and the delta cell index (ΔCI) was calculated for the baseline. CI data were analyzed and the slope of the curve was calculated by RTCA software v1.2.

7. *In vitro* DC stimulation

The role of H_4R in cytokine production of DCs was measured by an *in vitro* assay. Spleen-derived DCs were plated to 12 well plates ($3x10^6$ cells/well). With the exception of the control wells, DCs were incubated with 1 µg/ml LPS (24 hours, 37 ºC). Before LPS stimulation, cells were pretreated either with a H_4R agonist (4MH, 0.1 µM) alone for 15 min or with a H_4R antagonist (JNJ7777120, 10 µM) for 15 min prior to the agonist treatment. After incubation the cells were processed for RNA isolation and subsequent QRT-PCR analysis.

8. *In vivo* DC stimulation

WT and $H_4R^{-/-}$ mice were injected intra-peritoneally with either 200 µl CFA emulsion or 200 µl PBS. After 7 days of immunization, mice were sacrificed and spleens were removed for DC isolation. The obtained $CD11c^+$ DCs were used for RNA preparation followed by reverse transcription and QRT-PCR analysis.

9. Quantitative real-time PCR (QRT-PCR)

RNA isolation was performed with RNeasy® Mini Kit according to the manufacturer's instructions, then 2 µg total RNA was reverse transcribed using random primers. Relative quantification of target mRNA was performed with a TaqMan real-time RT-PCR assay on an ABI Prism 7000 Sequence Detection System following the manufacturer's instructions. Taqman probe sets were used as follows: TNF-α, IL-1ß, IL-6, IL-4, IL-10, Gata-3, IL-12, IFNγ, T-bet, CCR7, CCL19 and GAPDH as housekeeping internal control. GAPDH-normalized signal levels were calculated using the comparative C_t method.

10. Western blot analysis

The analyzed samples contained 15 µg protein and were incubated in loading buffer, denatured and the protein bands were separated by SDS-PAGE depending on the protein size and then transferred to PVDF membrane. After blocking with 4% BSA, membranes were washed and probed with primary antibodies. The Ag-Ab complexes were labeled with appropriate horseradish peroxidase-conjugated (HRP) secondary antibodies and visualized by a chemiluminescent detection system on Retina XBM x-ray film.

11. Statistics

Statistical analysis was performed using analysis of variance (ANOVA), as appropriate, and Tukey test as post hoc test. Statistical significance (p value) less than 0.05 was considered as statistically significant. The Statistica program version 8 was used for statistical analysis except adhesion studies where Origin 7.0 software was applied.

Human studies

1. Monocyte separation and *in vitro* differentiation of DCs

Peripheral blood mononuclear cells (PBMC) were obtained by Ficoll gradient centrifugation. $CD14^+$ monocytes were isolated from PBMC by immunomagnetic cell separation using anti-CD14-conjugated microbeads. To obtain iDCs $CD14^+$ monocytes (2×10^6 cells/ml) were cultured in serum-free AIMV medium supplemented with antibiotics, 100 ng/ml IL-4 and 80 ng/ml GM-CSF. At day 2, the same amount of cytokines was added and the cells were cultured for another 3 days. Resting DCs were harvested on day 5, or were activated by LPS (100 ng/ml) and IFNγ (10 ng/ml) and harvested on day 6.

When indicated, histamine (10 µM) or histamine combined with specific HR inhibitors (10 µM) were added at day 0 and day 2 together with the differentiating cytokines. The following HR antagonists were used: pyrilamine (H_1R, Sigma-Aldrich), famotidine (H_2R), JNJ7777120 and JNJ10191584 (H_4R).

2. Phenotypic characterization of DCs

Phenotyping of resting and activated DCs was performed by flow cytometry using anti-CD83-FITC and anti-CD1a-PE antibodies and isotype-matched control Ab. For measuring H_2R expression indirect staining was applied. Fluorescence intensities were measured by FACS Calibur and data were analyzed by the FlowJo software. The $CD1a^-$ and $CD1a^+$ cells were separated with FACS DiVa high-speed cell sorter.

3. Quantitative real-time PCR

Total RNA was isolated from DCs by Trizol reagent. Reverse transcription was performed from 100 ng total RNA using the High Capacity cDNA Archive Kit. QRT-PCR for the HRs, MMP-9 and MMP-12 genes was performed by ABI PRISM 7900 using Taqman gene expression assays. The comparative Ct method was used to quantify transcripts relative to the endogenous control genes GAPDH or 36B4.

4. Cytokine measurements

Culture supernatants of mature DCs were harvested 24 hours after LPS+IFNγ treatment and the concentration of IL-6 and IL-10 was measured by using OptEIA kits.

5. Migration assay

DCs were suspended at 10^6 cells/ml migration medium (0.5 % BSA in RPMI). The MIP-3β chemokine, diluted at 200 ng/ml in migration medium was added to the lower chambers in a final volume of 600 µl. DCs were added to the upper chamber in a final volume of 250 µl and the chemotaxis assay was conducted for 4 hours (37 ^oC). The number of transmigrated cells was counted by flow cytometry using polystyrene standard beads.

6. Chemokine array

Chemokine gene expression profiling of $CD1a^-$ and $CD1a^+$ DCs was studied with the Human Chemokines & Receptors PCR Array in accordance with the manufacturer's recommendations. Briefly, total RNA was isolated using RNeasy Mini Kit. After DNase I digestion, first strand cDNA synthesis was performed by using the RT^2 First Strand Kit. Real-time PCR measurement was performed on the ABI Prism 7000 real-time PCR platform. After cycling with real-time PCR, the amplification data were analyzed and statistical significance was calculated by SABiosciences on-line software.

Results

Mouse studies

1. HR expression of mouse splenic DCs

First, HR expression was tested at the protein level in splenic $CD11c^+$ DCs by Western blot and flow cytometry. As H_3R expression could not be detected by real-time PCR this receptor was not tested. H_1R was detected at 55 kDa, H_2R at 40 kDa, while H_4R showed a 44 kDa band by Western blot. Flow cytometric analysis also confirmed the cell surface expression of H_1R, H_2R and H_4R on splenic DCs.

2. Changes in HR expression during DC differentiation and activation

We monitored HR expression of bone marrow-derived $CD11c^+$ DCs on days 6, 8, 10 and 13 of *in vitro* differentiation by flow cytometry. H_1R expression was not found to remarkably change, while H_2R and H_4R expression was significantly reduced during the course of DC development. When DCs were treated with LPS on day 13 and tested for HR expression 24 hours later by flow cytometry we observed that H_2R expression was not altered, while the surface expression of H_4R was significantly elevated as a result of LPS activation.

3. The *in vitro* effect of histamine on H_4R and the peptide-presenting function of DCs

In the following experiments the impact of histamine was investigated on some relevant DC functions. Using an *in vitro* assay first we tested the effect of histamine on the peptide-presenting capacity of splenic $CD11c^+$ DCs. WT DCs were co-cultured with the aggrecan-specific 5/4E8 T cell hybridoma cells in the presence or absence of human aggrecan peptide for 24 hours. Histamine was added to the co-cultures at different concentrations. As a result of peptide-specific activation hybridoma cells produced IL-2 cytokine in the range of 4000-10000 pg/ml, while in the absence of antigen T cell activation did not occur. To verify that the activation of IL-2 production by histamine is not a direct effect on T cells, control wells containing only 5/4E8 cells, antigen and different concentrations of histamine were also set up where IL-2 levels remained undetectable. These results suggested that histamine could significantly reduce IL-2 secretion of T cells *in vitro*.

As a next step we investigated whether H_4R played role in the histamine-mediated effect on antigen presentation. DCs were treated with different concentrations of the H_4R agonist 4-MH and the level of IL-2 was detected. Among the concentrations tested, 100 µM 4-MH could significantly reduce the antigen presenting capacity of DCs. The role of histamine was further confirmed by using the specific receptor blockers famotidine for H_2R and JNJ10191584 for H_4R both added at 10 µM 1 hour before histamine treatment.

Antagonist-treated wells served as controls. We found that the H_2R antagonist further reduced the antigen presenting capacity of DCs, while the H_4R blocker restored the histamine-mediated reduced antigen presentation. These results indicated that the H_4R specifically affected the histamine-mediated reducing effect on DC peptide presentation.

In the next experiment peptide presentation of WT and $H_4R^{-/-}$ DCs was compared. We found that DCs that do not express functional H_4R on their surface possessed significantly higher antigen presenting capacity than WT cells.

4. Comparison of the expression of costimulatory molecules in WT and $H_4R^{-/-}$ DCs

Several data revealed that the pattern of surface molecules on DCs' membrane has an important role in T cell activation. Thus, we investigated whether WT and $H_4R^{-/-}$ DCs differed in the cell surface expression of characteristic DC proteins and costimulatory molecules and thus could be responsible for altered peptide presentation and T cell-derived IL-2 production. First WT and $H_4R^{-/-}$ histamine-treated (4 and 19 hours) or untreated splenic DCs were studied by flow cytometry. Neither the expression of the DC marker CD11c, nor MHCII and the costimulatory molecules CD40, CD80, CD86 was altered significantly in the presence of histamine when compared in the two animal groups. Next, *in vitro* generated DCs were applied, to which different concentrations of histamine were added on day 13 of differentiation, then 24 hours later the characteristic surface- and costimulatory molecules were measured by flow cytometry. Surface expression of the molecules tested did not differ significantly between WT and $H_4R^{-/-}$ DCs or after histamine treatment. Based on these results the differences found in the antigen presenting capacity of DCs could not be attributed to changes in cell surface molecule expression of the cells.

5. Role of histamine and H_4R in *in vitro* migration of DCs

The role of histamine and H_4R in the migration of splenic- or bone marrow-derived DCs was investigated with the help of an *in vitro* migration assay. First the effect of different concentrations of histamine or 4-MH was tested on splenic DC chemotaxis in Transwell system. The number of transmigrated cells was detected by flow cytometry normalized to a defined number of microparticles. Neither different concentrations of histamine, nor the H_4R agonist 4-MH influenced significantly DC migration.

Then we hypothesized that prolonged histamine treatment, if applied during DC differentiation may induce changes in the migratory capacity of the cells. To investigate this possibility DCs were differentiated in the presence or absence of 1 µM histamine added on days 0 and 3; 6 and 8 or 0, 3, 6 and 8 of *in vitro* cultures. On day 10, the migration of DCs

was studied in Transwell system. We observed a trend of stimulated DC migration by histamine in a time-dependent manner, but this increase was not statistically significant.

Finally, migration of WT and $H_4R^{-/-}$ DCs tested on day 10 of bone marrow-derived DC differentiation was also compared. DCs derived from H_4R-deficient mice showed significantly lower migration capacity than their WT counterparts.

6. Adhesion studies

To ascertain the functional background of the difference between WT and $H_4R^{-/-}$ DC migration adhesion studies were performed. Differentiated DCs were plated onto 96 well plates supplied with special electrodes suitable for measuring cell adhesion. After plating, the cells attach and spread relatively fast, which is detected as a steep increase of CI values. The adhesion curve of DCs treated with 1 µM histamine showed similar slope with the control, albeit with a faster kinetic. 2 hours after seeding the cells addition of histamine induced a significant increase in CI value as compared to the control.

In the next experiment we questioned if a chronic histamine challenge during DC differentiation may influence adhesion of the cells. Cells were treated with different concentrations of histamine on day 0, 3, 6 and 8 of differentiation and cell adhesion was measured on day 10. Histamine in both concentrations could enhance CI values compared to the control, however pretreatment with 1 µM histamine was more effective in increasing cell adhesion than the 10 µM concentration. Comparing the slopes of the adhesion curves demonstrated that 1 µM histamine applied during DC differentiation generated a faster and on the whole an increased adhesion, which could not be influenced by additional histamine applied right before the experiment.

When we compared the adhesive properties of WT and $H_4R^{-/-}$ DCs, cells of different genotypes had similar adhesion characteristics, but in the first period (72 min) the average CI of $H_4R^{-/-}$ cells showed a faster increase than that of the WT cells. Five hours after seeding the difference of the two cell types was diminished. The fast phase of cell adhesion is also characterized by the slopes of the curves, and showed significant difference of the two investigated DCs; the $H_4R^{-/-}$ curve had increased CI value. About 120 min after seeding, the CI showed significantly different values in WT and $H_4R^{-/-}$ cells, respectively.

To test whether H_4R is the HR responsible of the difference shown before, WT DCs were treated with H_1R, H_2R and H_4R antagonists in the presence or absence of histamine and cell adhesion was measured. Blocking of H_1R and H_2R did not result in a considerable change as compared to control or histamine treated cells, while the H_4R antagonist JNJ10191584 could effectively reduce the increasing effect of histamine on DC adhesion.

7. Effect of H_4R on cytokine production of *in vitro* stimulated DCs

In our *in vitro* system we investigated the cytokine response of activated splenic DCs after H_4R stimulation. Separated DCs were pretreated with the H_4R agonist 4-MH for 15 min followed by LPS activation for 24 hours. In order to clarify the role of H_4R, we used the specific antagonist JNJ7777120, added to cells 15 min before agonist challenge. Real-time PCR measurements revealed that the mRNA expression of IL-1ß, one of the paramount inflammatory cytokines, was elevated after a combined treatment with a H_4R agonist and LPS and this significant effect could be reversed by the H_4R blocker JNJ7777120. Importantly, no significant alteration was observed in the expression of the IL-6, IFNγ, IL-10, IL-12a cytokines and the CCL19, CCL2, CCL4, CCR7 chemokines after H_4R stimulation.

8. Cytokine responses of *in vivo* activated WT and $H_4R^{-/-}$ DCs

Most data on DC cytokine production are based on the results of *in vitro* experiments. To obtain more information about DC cytokine expression, we carried out an *in vivo* assay and compared the cytokine profiles of CFA stimulated WT and $H_4R^{-/-}$ DCs. On day 7 after injection, we monitored the expression of various cytokines, chemokines and major transcription factors involved in Th1, Th2 responses and Treg functions of isolated splenic DCs by real-time PCR. We detected significantly lower IL-10, Gata-3, IL-12 and IFNγ mRNA expression in $H_4R^{-/-}$ DCs as compared to WT controls. Among the genes tested, CFA treatment reduced the expression of IL-1ß, IL-4, IL-10, CCL19 and CCR7 significantly, regardless of the genotype of the cells. We also found genotype-dependent differences in response to CFA; CFA treatment reduced IL-6, Gata-3, and T-bet expression in WT animals, but it was ineffective in the H_4R deficient DCs. In KO mice IFNγ mRNA levels were elevated by *in vivo* CFA injection in contrast to WT animals where it remained unaltered.

Human studies

1. Histamine modulates monocyte-derived DC differentiation

In our human studies first we tested how histamine, potentially acting through different HRs can modulate the differentiation of the previously identified $CD1a^-$ and $CD1a^+$ DC subsets. Monocyte-derived DCs were generated by IL-4 and GM-CSF cytokine combination in the presence or absence of histamine applied in the course of cell differentiation on day 0, 2 and 5. Flow cytometric measurements revealed that histamine significantly reduced the proportion of $CD1a^+$ DCs as compared to cells generated in the absence of histamine. To identify the HR involved in this effect, specific pharmacological blocking agents were used during the differentiation process. We found that the presence of

the H_2R antagonist famotidine could prevent the reducing effect of histamine on $CD1a^+$ cell differentiation. The H_1R antagonist pyrilamine further reduced the ratio of $CD1a^+$ cells, whereas both H_4R antagonists (JNJ7777120 and JNJ10191584) had no or only marginal effects. The inhibitory effect of histamine on $CD1a^+$ cell differentiation was confirmed at the protein level by flow cytometric analysis showing the cell surface expression of H_2R on $CD1a^-$ and $CD1a^+$ DC subsets and also the efficient blocking of this effect by famotidine, but not by the other inhibitors.

2. Expression of HRs in $CD1a^-$ and $CD1a^+$ DC subsets

The expression of HRs by human DCs has previously been demonstrated however, their expression in the phenotypically and functionally distinct $CD1a^-$ and $CD1a^+$ DC subsets has not been investigated so far. To assess the DC subset-specific expression of HRs in *in vitro* differentiated LPS+IFNγ-activated monocyte-derived DCs the cells were sorted to $CD1a^-$ and $CD1a^+$ subpopulations by flow cytometry and H_1R, H_2R and H_4R expression was compared by QRT-PCR. H_3R expression could not be detected, H_1R expression was significantly higher in $CD1a^-$ DCs, but H_4R was higher in $CD1a^+$ cells as compared to their $CD1a^-$ counterpart, whereas H_2R was expressed by both subsets at high levels. This finding was also supported by the detection of H_2R by flow cytometric analysis on the surface of both $CD1a^-$ and $CD1a^+$ cells at comparable levels.

3. Effect of histamine on the activation of $CD1a^-$ and $CD1a^+$ DC subsets

Previously we described that inflammatory cytokines can block the transition of $CD1a^-$ cells to $CD1a^+$ DCs and thus stabilize the $CD1a^-$ phenotype for further activation. To monitor the effect of histamine on the activation of $CD1a^-$ and $CD1a^+$ subsets, DCs were generated in the presence of histamine and activated by the combination of LPS and IFNγ. Cell surface expression of CD1a and the activation marker CD83 was monitored in the $CD1a^-$ and $CD1a^+$ DC subsets by flow cytometry. The results showed that histamine did not affect significantly the expression of CD83, and both the $CD1a^-$ and $CD1a^+$ subsets could be activated by LPS+IFNγ. Histamine, present in the course of *in vitro* monocyte-derived DC differentiation, was able to reduce the proportion of $CD1a^+$ cells and consequently decreased the ratio of both resting and activated $CD1a^+CD83^+$ cells, while the $CD1a^-CD83^+$ fraction became larger only in mature DCs. In line with our results obtained with resting DCs the H_2R antagonist famotidine suspended this histamine-mediated effect, while the H_1R antagonist pyrilamine further reduced the ratio of $CD1a^+CD83^+$ cells. These results suggest that histamine modulates DC activation through its inhibitory effect on $CD1a^+$ DC differentiation.

DCs are sensitive to changes in their environment and thus can be activated by various stimuli, which result in the secretion of cytokines and chemokines. To test whether histamine or HR-specific antagonists can modify the cytokine production of DC subsets we measured the concentrations of cytokines in the culture supernatant of activated $CD1a^-$ and $CD1a^+$ DC cultures by ELISA. Statistically significant increase of the secreted pro-inflammatory cytokine IL-6 and the regulatory cytokine IL-10 could be shown when DCs were generated in the presence of histamine. This effect also could be inhibited by famotidine indicating again the involvement of H_2R in this process. The secretion of IL-12p70 was detected preferentially in DCs with high, >60% of $CD1a^+$ cell content and its production could not be consistently inhibited by famotidine.

5. Effect of histamine on DC migration

Migration is an important and indispensable feature of DCs. Histamine not only induces cell migration mediated by different HRs, but at the same time it is also able to stimulate matrix degrading enzyme production. Our *in vitro* migration experiments revealed that the presence of histamine during monocyte-derived DC differentiation significantly enhanced the migratory potential of DCs that could be shown at the level of both spontaneous and MIP-3β-induced migration. Famotidine, the specific H_2R antagonist could reverse this effect pointing to the role of H_2R in the modulation of cell mobility. Matrix degrading enzymes are also essential components of cell migration. Monitoring mRNA expression of MMP-9 and MMP-12 by QRT-PCR showed that the presence of histamine was able to up regulate the mRNA expression of these matrix degrading enzymes in both resting (iDC) and activated (mDC) DCs. This effect could also be reversed by famotidine further supporting the role of H_2R in regulating DC migration.

6. Histamine-induced expression of C5a receptor is restricted to the $CD1a^-$ DC subset

In the next set of experiments we performed a global screening using a Q-PCR-based array to identify chemokines and chemokine receptors involved in the histamine-induced H_2R-mediated enhancement of DC migration. Following LPS+IFNγ activation, the monocyte-derived DCs generated in the presence of histamine, famotidine, or histamine together with famotidine were separated to $CD1a^-$ and $CD1a^+$ subsets and their chemokine and chemokine receptor expression profiles were determined. When DCs were generated in the presence of histamine, increased mRNA expression of C5aR1 was detected in the $CD1a^+$ subset and famotidine abolished this effect. However, C5aR1 protein expression clearly showed that its expression was dramatically upregulated by histamine preferentially in the $CD1a^-$ subset at both their resting and activated differentiation states. As famotidine could completely abolish

the effect of histamine, these results also revealed the H_2R dependency of this histamine-induced effect.

In addition to identifying C5aR1 as a histamine-induced chemotactic receptor of DCs preferentially expressed by the $CD1a^-$ subset, we also identified further migration-related genes. The expression of CXCR4 and CX3CR1 also increased significantly in DCs generated in the presence of histamine and this effect on CX3CR1 could be inhibited by famotidine in the $CD1a^+$ DC subset, while in $CD1a^-$ cells histamine did not exert this modulatory effect.

Discussion

DCs represent a minor population of leukocytes but act as important sentinels and are characterized by diverse biological functions. Beside capturing, processing and presenting antigens, they are capable of regulating the direction, quantity and quality of effector mechanisms that serve for the elimination of invading organisms and dangerous self structures. Through the activation of antigen-specific T lymphocytes and the production of cytokines and chemokines DCs act as a functional bridge between innate and acquired immunity.

In this PhD work we investigated the functional cross-talk of histamine with DCs in two experimental systems. The murine system offered means to compare the effects of histamine on DCs in genetically modified strains *in vivo* and by using tissue-derived DCs *in vitro*. The human system offered us to analyse the effects of histamine on DC subtype differentiation and functions.

The size of the HRs has been shown to vary in a wide range depending on the tissue, cell type, post-translational modification and detection method. In our hands immunoblot and flow cytometric analysis certified the expression of H_1R, H_2R and H_4R in mouse splenic DCs. Beside the H_4R protein band detected by Western blot the other higher molecular mass band could correspond to the glycosylated form of the receptor.

DC differentiation is a complex process regulated at multiple levels. We observed no change in the expression of H_1R, whereas that of the H_2R and H_4R decreased significantly in the course of *in vitro* differentiation of bone marrow-derived DCs. LPS did not alter H_1R or H_2R protein expression, however H_4R expression was elevated significantly by LPS stimulation. It was demonstrated that during *in vitro* differentiation of DCs from human monocytes the expression of H_1R and H_3R reduced, while that of the H_4R increased. Others showed higher expression of H_4R in resting than in activated $CD4^+$ and $CD8^+$ T lymphocytes. Based on these data we suggest, that since different HRs activate distinct signaling cascades, the observed alterations in HR expression may modify the strength and the direction of histamine-mediated effects depending on the life cycle or activation state of the cells.

Antigen presentation is of crucial importance in the activities of DCs, still a few data are available on the effects of histamine on basic DC functions. We found concentration-dependent, biphasic and significantly decreased antigen presentation of murine splenic DCs in the presence of histamine. We identified H_4R as the regulator of this process, as its agonist (4-MH) similarly to histamine declined the peptide presenting function of DCs, while the H_4R

antagonist JNJ10191584 almost completely reversed this effect. The observation that significantly higher amounts of IL-2 were produced when the 5/4E8 T cell hybridoma cells were stimulated by peptide-loaded DCs of H_4R-deficient mice than DCs from WT controls however, further indicated the role of H_4R in this histamine-mediated effect. We also found that long-term H_4R deficiency had a more pronounced impact on antigen presentation than short-term antagonist treatment. These data suggest that histamine exerts this effect through H_4R and it can evolve both acutely during antigen presentation and also in the course of DC development.

The efficacy of antigen presentation can be influenced by several factors, such as the subtype of DCs, the mechanism of antigen uptake and processing and the expression of cell surface costimulatory molecules. The molecular mechanism, by which histamine acts on antigen presentation via H_4R remains unknown, since we could not detect any differences in either MHC class II or costimulatory molecule expression of histamine-treated $H_4R^{-/-}$ and WT splenic DCs. IFNγ has been shown to affect certain components of the antigen presentation machinery. Since we found that the induction of IFNγ production was more pronounced in H_4R-KO DCs as compared to the WT ones, enhanced IFNγ levels may account for the elevated T cell-stimulatory capacity of H_4R-deficient DCs.

Several factors including chemokines, cytokines and small bioactive molecules like histamine may influence the migratory and chemotactic properties of DCs and histamine has long been known to influence the migration of DCs. Before the discovery of H_4R chemotactic effects provoked by histamine were attributed primarily to H_1R and H_2R. Later, the migration regulatory effect of histamine through H_4R of different immune cell types such as eosinophils, mast cells, γð T cells and natural killer cells was demonstrated. The involvement of H_2R and H_4R was shown in human DCs, while in murine differentiated DCs the role of H_1R and H_4R was shown.

In our migration studies we found that neither different concentrations of histamine nor 4-MH could induce a significant alteration in splenic DC migration *in vitro*. Supposing that spleen-derived DCs are less susceptible to acute histamine stimulus than freshly differentiated cells, we monitored the effect of a long-term histamine treatment on DC migration. We found that the length of histamine treatment during *in vitro* DC differentiation correlated to migrating cell numbers. Comparing the migratory capacity of WT and $H_4R^{-/-}$ DCs we concluded that DCs generated in the absence of H_4R possessed a significantly reduced migration capacity then WT cells, suggesting the role of H_4R in this process. Up to now only one paper has been published in which the effect of histamine on mouse DC

migration via H_1R and H_4R was demonstrated. However, we have to notice that in most cases histamine and HR ligands were applied in much lower concentration than the above (1mmol/l), physiologically not available concentration.

Adhesion is an indispensable step of cell migration. We were the first to use an impedance-based xCELLigence RTCA SP system to study the adhesive capacity of murine differentiated DCs. Histamine significantly increased DC adhesion when presented transiently or for a long period of time. We identified H_4R as the receptor responsible for this histamine-mediated effect, as comparison of the adhesive capacity of WT and $H_4R^{-/-}$ DCs revealed that both parameters characterizing the adhesion of the cells, i.e. slope and ΔCI, were significantly higher in H_4R-KO than in WT cells. These results were surprising since previously we detected impaired migration of H_4R-deficient DCs. As adhesion is a prerequisite of cell migration, the above processes were expected to show direct correlation. It has recently been published that murine DC migration could be negatively regulated by adhesion. The involvement of H_4R was further confirmed by *in vitro* H_4R antagonist treatment showing that JNJ10191584 could effectively reduce the increasing effect of histamine on DC adhesion. Further experiments are required to obtain more molecular data on the action of histamine via H_4R governing adhesion and migration however, these results revealed the H_4R dependency of this histamine-induced effect.

Cytokines and chemokines secreted by activated DCs are crucial components of the modulation of immune processes. The repertoire of soluble cytokines produced by DCs is associated with the developmental stage or maturation status of the cells and are important in inducing an effective primary immune response and several lines of evidence support that histamine influences cytokine production of DCs. Before the discovery of H_4R, mainly H_2R was found to be involved in histamine-induced Th2 polarization of DCs. Increasing body of evidence support that Toll-like receptor signals in DCs not only enhance the endocytosis of antigens but also influence the immunological outcome of their interactions with T cells. The pro-inflammatory cytokine IL-1ß is a crucial mediator of the innate immune system and beside activated mononuclear phagocytes, DCs are also important sources of this cytokine. Mazzoni *et al*. have shown that histamine blocked LPS-induced IL-1ß expression in immature human DCs. In this experimental system the general receptor activator histamine was used, thus the results gained are possibly reflect the accumulated effect on different HRs.

In our work IL-1ß mRNA expression was tested using either H_4R agonist or antagonist treatment followed by LPS stimulation. Our results revealed that the LPS-induced expression of IL-1ß was further induced by the H_4R agonist 4-MH, while the H_4R antagonist

JNJ7777120 effectively reversed this effect demonstrating the participation of histamine via H_4R in governing IL-1ß expression.

Most data on DC cytokine production is based on the results of *in vitro* studies. In order to get more insight into its importance, we applied an *in vivo* assay in which WT and $H_4R^{-/-}$ animals were stimulated by CFA and then the cytokine profile of DCs was compared. $H_4R^{-/-}$ DCs showed significantly altered mRNA expression of some Th1 and Th2 cytokines. Most papers based on *in vitro* data report the elevation of Th2 and reduction of Th1 cytokines, which mediated principally through H_2R. Gutzmer and co-workers detected reduced IL-12p70 production in human monocyte-derived DCs, while IL-10 expression was not altered after histamine treatment. This effect was mediated by H_2R and H_4R. In line with previous data we could not detect any changes in TNF-α expression (neither after treatment nor in the genetically different mice). CFA treatment showed genotype-dependent effects in case of some molecules; it decreased IL-6, Gata-3 and T-bet mRNA expression in WT DCs, while it proved to be inefficient in $H_4R^{-/-}$ DCs. Our results on IL-6 are contradictory to the data published by Dunford *et al.*. Applying different activation stimuli *in vitro*, they measured decreased IL-6 protein expression after H_4R blockade. $H_4R^{-/-}$ DCs produced more IFNγ than WT ones following CFA treatment. We detected similar difference in IFNγ expression when comparing WT and $HDC^{-/-}$ DCs.

In the second part of the studies we investigated the effects of histamine on the differentiation and activities of two, developmentally related and *in vivo* relevant human monocyte-derived DC subsets generated from $CD14^{high}$ monocytes. We found that i) histamine modulated DC differentiation by supporting the development of $CD1a^-$ DCs; ii) this effect was attributed to H_2R as only its specific synthetic inhibitor could restore the generation of $CD1a^+$ cells; iii) H_2R was highly expressed by both DC subsets, whereas H_1R and H_4R were expressed in a subset-specific manner; iv) histamine modulated DC activation through its inhibitory effect on $CD1a^+$ DC differentiation; v) histamine-induced reduction of $CD1a^+$ DCs resulted in increased secretion of IL-6 and IL-10; vi) histamine modulated the expression of C5aR1 in a H_2R and subset specific manner; vii) histamine induced spontaneous and chemokine-mediated DC migration of both subsets and modulated the production of the MMP-9 and MMP-12 enzymes also involved in the regulation of DC migration.

DC differentiation is a complex and highly regulated process driven by the actual tissue environment of the cell. Lipids and lipoproteins have previously been identified as modulators of the cell surface expression of type I and type II CD1 proteins, which act as both presenting molecules of microbial glyco- and phopholipids and also as phenotypic markers of

human DC subsets. The modulatory effect of histamine on the expression of CD1a and the *in vitro* differentiation of DCs has previously been reported. Here we not only confirmed these findings, but extended our studies to demonstrate that histamine has an impact on other DC functions which may be regulated in a subset-specific manner.

DC functions may be associated with more than one HR, thus we sought to generate monocyte-derived DCs *in vitro* in the presence of histamine or histamine in combination with pharmacological antagonists of H_1R, H_2R and H_4R. As famotidine, a potent antagonist of H_2R was the only compound that could prevent the histamine-mediated inhibition of $CD1a^+$ DC differentiation we identified H_2R as the receptor involved in this regulation.

Several research groups demonstrated the expression HRs in human DCs however, their expression in $CD1a^-$ and $CD1a^+$ cells has not been analyzed so far. Similar to previous results we could not detect H_3R expression in DCs but found high expression of H_2R in both $CD1a^-$ and $CD1a^+$ DCs. Interestingly, H_1R and H_4R mRNA expression showed a subset-dependent pattern: $CD1a^-$ DCs expressed significantly higher levels of H_1R than $CD1a^+$ cells, whereas H_4R expression was significantly higher in the $CD1a^+$ DC subset. This finding raises interesting questions concerning the role of H_4R in a situation where due to the presence of histamine the generation of the cell type i.e. $CD1a^+$ DCs carrying the receptor at high levels is inhibited. Based on this scenario and taken the previously described inflammatory nature of $CD1a^+$ DCs in individuals with high $CD1a^+$ DC ratios may develop more severe inflammation in the presence of histamine than those with low $CD1a^+$ numbers. Alternatively, this expression pattern may have a regulatory function to keep histamine-mediated regulation under check.

DC maturation is initiated by the engagement of different surface receptors and results in phenotypic and functional changes. Histamine was shown to induce transient up-regulation of MHC class II proteins and the costimulatory molecules CD40, CD80 and CD86 in human monocyte-derived DCs and antagonists of both H_1R and H_2R prevented histamine-induced CD86 expression but did not modify CD40 expression. When DCs were activated with LPS+IFNγ we found that due to the histamine-induced reduction of $CD1a^+$ DC numbers the ratio of $CD1a^+CD83^+$ cells decreased, while the $CD1a^-CD83^+$ cell fraction became more prominent. This observation demonstrates that histamine, by counteracting $CD1a^+$ DC differentiation has an impact on the DC subset distribution of mature DCs in a H_2R-dependent manner.

The hallmark of activated DCs is the production of cytokines and chemokines, which may act in an autocrine or paracrine manner. Previous results demonstrated that histamine

induced altered cytokine production in DCs, supported polarization of T lymphocytes to Th2 direction, and the cytokine profile of resting $CD1a^-$ and $CD1a^+$ DCs was different. In line with these findings we also observed that upon activation by CD40L or TLR ligands both $CD1a^-$ and $CD1a^+$ DCs secreted pro-inflammatory cytokines but IL-12p70 and CCL1 production was mainly attributed to the $CD1a^+$, whereas IL-10 secretion to the $CD1a^-$ subset. DCs generated in the presence of histamine and activated by LPS+IFNγ produced significantly higher levels of the pro-inflammatory cytokine IL-6 and the regulatory cytokine IL-10 as compared to cells generated in the absence of histamine. Again, famotidine was the only inhibitor that could interfere with this effect. In our studies IL-12p70 secretion was detected in DCs with high (>60%) $CD1a^+$ cell content and famotidine had no significant effect on cytokine secretion indicating that IL-12p70 secretion is not dependent on H_2R. In a previous study IFNγ was shown to up-regulate H_4R and its stimulation resulted in the down-regulation of IL-12p70 and CCL2 production in human monocyte-derived DCs.

Resting DCs migrate to both body surfaces and interstitial spaces where they encounter with self- or foreign antigens. After antigen challenge, migration of activated DCs through lymphatics ensures DC-T cell contact in lymphoid organs. Several independent research groups found that histamine or histamine agonists exerted chemotactic effects for resting, but not for activated DCs. By using specific receptor antagonists the involvement of both H_3R and H_1R has been demonstrated as modulators of cell migration. In a skin DC migration assay both histamine and a H_4R agonist induced enhanced chemotaxis, which could be blocked by both H_1R and H_4R antagonists. Similar results were obtained *in vitro* by using bone marrow-derived mouse DCs. Our previous results also verified the role of H_4R in murine DC migration.

In our present migration experiments histamine increased both spontaneous and MIP-3β-induced migration of DCs. Famotidine was the only HR antagonist that could reverse this effect. Histamine was also shown to stimulate the production of the matrix degrading enzymes and the histamine-induced increase of MMP-13 and MMP-3 production was shown *in vitro*. The essential role of MMP-9 in DC migration was measured in reconstituted basement membrane (Matrigel) and *in vivo* by migration to the draining lymph node and the expression of MMP-12 in DCs was also shown. Histamine induced up-regulation of MMP-9 and MMP-12 and their inhibition by the H_2R blocker famotidine confirmed the dominant role of H_2R in this DC function as well.

Migration of DCs in blood and lymph, and from peripheral tissues to secondary lymphoid organs is regulated at multiple levels and a number of cytokines, chemokines and

their receptors are implicated in the regulation of this complex process. The combination of chemokine receptors, induced in activated DCs when developed in the presence of histamine and stimulated with LPS+IFNγ involved C5aR1, CXCR4 and CX3CR1, previously identified in the sulpho-LacNAc expressing DCs (slanDCs/MDC-8), which represent the largest population of blood DCs. These circulating DCs are highly pro-inflammatory due to their production of TNF-α and IL-12p70, but in contrast to $CD1c^+$ conventional blood DCs they are negative for the CD11c, CD14 and CD1 markers. C5a is a pro-inflammatory mediator that has recently been detected in monocyte-derived DCs and signaling through C5aR1 has been found to regulate the development of Treg and Th17 cells. Here, we show for the first time that monocyte-derived DCs, differentiated in the presence of histamine and activated by LPS+IFNγ, preferentially give rise to $CD1a^-C5aR1^+$ cells.

CXCR4 endowed with potent chemotactic activity is up-regulated upon DC activation induced by LPS, TNF-α or CD40L and engagement by its specific ligand CXCL12 promotes DC activation, survival and chemotaxis both *in vitro* and *in vivo*. The CX3CR1 transmembrane protein has been suggested to be enhanced by IFNγ in TNF/iNOS-producing DCs differentiated from classical monocytes and it is involved in the recruitment of DCs by its specific ligand CX3CL1/fractalkine. Based on these results we propose that histamine, in combination with inflammatory signals is able to induce the expression of a typical combination of chemokine receptors that also modulate monocyte-derived DC functions. However, this effect can be further up-regulated in the presence of the CCL19/MIP-3β chemokine, which specifically attracts CCR7 expressing activated DCs.

The *in vivo* and *in vitro* studies on the role of histamine on murine and human DCs revealed that histamine mediated several cellular activities of both murine and human DCs. The absence of H_4R resulted in an enhanced antigen presentation capability and adhesion characteristics of mouse DCs. Furthermore, H_4R deficiency decreased mouse DC migration and gene expression of some cytokines as well as it altered the inducibility of cytokine mRNA production. We have to take into consideration that changes observed here, are more significant in the case of chronic lack of H_4R rather than in the acute inhibition of the receptor. This suggests that defect of H_4R may also influence DC functions both in a direct and indirect manner. Furthermore, our results demonstrated that histamine had a profound effect on the development of human $CD1a^+$ DCs that was mediated by H_2R. This regulation had a further impact on cytokine production known to be different in the $CD1a^-$ and $CD1a^+$ subsets. As H_2R was expressed at high levels in both DC subsets, it may dominate the regulation of multiple DC-related functional activities. In contrast, H_1R and H_4R with

opposing subset-related expression may have a regulatory or fine-tuning role in histamine-induced functional activities.

Histamine is not only the major mediator of acute inflammatory and immediate hypersensitivity responses, but has also been demonstrated to affect chronic inflammation and several essential functions of the immune system. It is also well known that DCs, which control homeostatic processes and regulate acute and chronic inflammation can be affected by this small biogenic molecule. Thus the knowledge about the molecular mechanism of histamine actions on DCs has crucial importance to develop effective therapeutic strategies for the treatment of inflammatory diseases.

As DCs may localize to the vicinity of histamine producing cells, such as basophils and mast cells present in an inflammatory environment, they are potential targets of histamine. The results gained in our study highlighted some interesting aspects of histamine and DC crosstalk, which may help in better understanding the underlying mechanisms of histamine-mediated conditions. Indeed, more research is needed to understand the complex role of DCs in histamine-mediated immune responses, however by every step we are closer to utilize the experimental results in clinical settings especially in drug design and development.

Publications

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List of publications related to the dissertation

1. **Simon, T.**, Gogolák, P., Kis-Tóth, K., Jelinek, I., László, V., Rajnavölgyi, É.: Histamine modulates multiple functional activities of monocyte-derived dendritic cell subsets via histamine receptor 2. *Int. Immunol. Epub ahead of print (2012)*
   DOI: http://dx.doi.org/10.1093/intimm/dxr107
   IF:3.301 (2010)

2. **Simon, T.**, László, V., Falus, A.: Impact of histamine on dendritic cell functions. *Cell Biol. Int. 35* (10), 997-1000, 2011.
   DOI: http://dx.doi.org/1042/CBI20100844
   IF:1.747 (2010)

3. **Simon, T.**, László, V., Lang, O., Buzás, E., Falus, A.: Histamine regulates relevant murine dendritic cell functions via H4 receptor. *Front Biosci (Elite Ed). 3*, 1414-1424, 2011. *

4. **Simon, T.**, Jelinek, I., Apponyi, G., László, V., Rajnavölgyi, É., Falus, A.: Expression and function of histamine H4 receptor in mouse splenic dendritic cells. *Inflamm. Res. 59* (Suppl.2), S201-S203, 2010.
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   IF:2.004

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4032 Debrecen, Egyetem tér 1. e-mail: publikaciok@lib.unideb.hu

List of other publications

5. **Simon, T.**, F. Semsei, Á., Ungvári, I., Hadadi, É., Virág, V., Nagy, A., S. Vángor, M., László, V., Szalai, C., Falus, A.: Asthma endophenotypes and polymorphisms in the histamine receptor HRH4 gene.
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PLoS One. 6 (8), e23653, 2011.
DOI: http://dx.doi.org/10.1371/journal.pone.0023653
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** In the editorial invitation received in 2009 the editor of Frontiers in Bioscience clearly referred to the impact factor of the journal (IF: 3.6 in 2008), however next year the journal divided into 3 sections and at calculation of IF in 2010 the new "Landmark edition" has already published less articles (IF: 4.048). Thus, regarding the article published in "Elite edition" no IF is available. The publication is included in the PubMed and Scopus, its type is "peer-reviewed original article".*

The Candidate's publication data submitted to the Publication Database of the University of Debrecen have been validated by Kenezy Life Sciences Library on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

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Posters and presentations at conferences:

Detection and function of H4R in spleen-derived dendritic cells
(European Histamine Research Society Conference, Fulda, 2009., poster presentation)

Detection and function of H4R in spleen-derived dendritic cells
(Semmelweis University PhD Days Conference, Budapest, 2009., poster presentation)

Detection and function of H4R in spleen-derived dendritic cells
(Hungarian Society For Immunology Conference, Harkány, 2009., poster presentation)

Role of histamine and its H4 receptor in murine dendritic cell functions
(European Histamine Research Society Conference, Durham, 2010., oral presentation)

A hisztamin és a hisztamin H4 receptor szerepe az egér dendritikus sejtek működésében
(Hungarian Society For Immunology Conference, Szeged, 2010., oral presentation)

A hisztamin és a hisztamin H4 receptor szerepe az egér dendritikus sejtek működésében
(Semmelweis University PhD Days Conference, Budapest, 2011., oral presentation)

Histamine modulates multiple functional activities of monocyte-derived dendritic cell subsets via histamine receptor 2
(World Immune Regulation Meeting VI. Conference, Davos, 2012., poster presentation)