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Histamine regulates relevant murine dendritic cell functions via H_4 receptor

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TABLE OF CONTENTS

1. ABSTRACT

Histamine, produced by dendritic cells (DCs) or by other cells of the immune system, may have significant impact on DC activities. We investigated the influence of histamine and histamine H_4 receptor (H_4R) on some relevant functions of DCs. Histamine significantly decreased the antigen presentation capacity of splenic DCs, and this effect was reversed by a H_4R antagonist. Furthermore, enhanced antigen presentation was detected in $H_4R^{-/-}$ DCs. Prolonged histamine treatment during DC differentiation stimulated migration, albeit the increase was not significant. H_4R-deficient DCs possessed significantly lower migration capacity than their wild-type counterparts. Monitoring *in vivo* and *in vitro* DC cytokine production revealed that a H_4R agonist in combination with LPS, increased IL-1 beta mRNA expression, and a H_4R antagonist reversed this effect. In H_4R-deficient mice we detected decreased mRNA expression of some DC-derived cytokines including IFN-gamma and IL-10. Upon CFA stimulation, genotype-dependent differences were found in the expression of IL-6 and IFN-gamma. Our data suggest that H_4R plays a crucial role in variety of functions of murine DCs.

2. INTRODUCTION

Dendritic cells (DCs) represent a heterogeneous fraction of rare hematopoietic cells, and are crucial components of the immune system. These bone marrow-derived cells function as professional antigen presenting cells (APC). DC progenitors are seeded through blood or lymphatic stream to lymphoid and nonlymphoid tissues where they develop into immature DCs. In the periphery, immature DCs are „sentinels" of the immune system, and are caracterized by a high ability to capture antigens. They process foreign antigens, and possess low T cell stimulatory capacity. After antigen challenge and/or stimulation with inflammatory mediators, DCs migrate to the secondary lymphoid organs in which they home to T cell areas. During their migration, they undergo dramatic changes in their phenotype and their functions are modulated. This process is referred to as DC maturation. DCs loose their ability to capture antigens and acquire an increased capacity to activate naive T cells. They express increased levels of cell surface proteins involved in T cell stimulation, such as costimulatory and MHC class II molecules. They produce a large variety of cytokines and chemokines that also favor T cell activation and polarization.

It is well documented that local environmental factors such as cytokines, chemokines and a large number of inflammatory mediators like histamine, have remarkable influence on maturation as well as on the functions of DCs (1). Histamine is a multifunctional small-sized biogenic amine that is stored abundantly in, and is rapidly released from some immune cells like mast cells and basophils found in the vicinity of DCs. It was shown that DCs themselves were sources of histamine: they both synthesize and release histamine (2, 3). A variety of physiological and pathological functions of histamine are mediated by four different receptors: H_1R, H_2R, H_3R and H_4R. H_4R, similarly to all other histamine receptors, is a constitutively active, G protein-coupled receptor expressed mainly on immune cells including mast cells, monocytes, eosinophils, basophils, DCs, T cells and natural killer cells (4, 5). However, recently it was demonstrated that H_4R was functionally expressed also on neurons in the mammalian central nervous system (6). H_4R is considered to play a role in a number of inflammatory disorders such as allergy, asthma, chronic pruritus and autoimmune diseases (7). Lately, the involvement of H_4R was demonstrated in some DC functions (8, 9).

In our present study which was carried out to obtain more data about the role of H_4R in murine DC regulation we used H_4R-deficient ($H_4R^{-/-}$; H_4R-KO) mice and specific H_4R antagonists.

3. MATERIALS AND METHODS

3.1. Mice

$H_4R^{-/-}$ mice were generated by Lexon Genetics, and were kindly provided by Dr. Robin L. Thurmond (Johnson & Johnson Pharmaceutical Research and Development, San Diego, CA2121). Mice were crossed on to BALB/c background for seven generations in our laboratory. Homozygous wild-type controls (WT) of the same backcross number and generation were used as controls. For all experiments, mice were maintained under specific pathogen-free conditions, at constant temperature (22 °C) and under a light cycle of 12-h light/12-h darkness in groups of 10. Food and water were freely available. Animals were used at 8–12 weeks of age. All experiments were carried out with the approval of the local ethics committee. The authors adhered to the Declaration of Helsinki and the IASP for the care and use of animals throughout the study.

3.2. Isolation of DCs from spleen

DCs were purified from spleens of $H_4R^{-/-}$ and WT mice after collagenase D digestion (Roche Diagnostics, Mannheim, Germany). CD11c+ cells were obtained by immunomagnetic bead selection with CD11c-MACS beads (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of cells was checked by flow cytometry, and the frequency of CD11c+ DCs was ~ 95% in all experiments.

3.3. DC differentiation in bone marrow cultures

The procedure used in this study was first described by Lutz *et al.* (10). Briefly, bone marrow was flushed from tibiae of the mice with PBS. Clusters within the suspension were disintegrated by vigorous pipetting. After washing, $2x10^6$ cells in RPMI-1640 (Sigma-Aldrich, Deisenhofen, Germany) with 200 U/ml rmGM-CSF (R&D Systems Inc. Minneapolis, USA) were transferred to bacteriological Petri dishes with 100 mm diameter. At day 3 another 10 ml medium containing 200 U/ml rmGM-CSF was added to the plates. At days 6 and 8, half of the tissue culture supernatant was collected and centrifuged. The cell pellet was resuspended in 10 ml fresh medium with rmGM-CSF, and put back into the original plate. At day 10 cells were used for experiments. The purity was checked by flow cytometry. The frequency of CD11c+ DCs was between 85% and 95% in all experiments.

For DC differentiation RPMI-1640 was supplemented with Penicillin (100 U/ml, Invitrogen, Gibco, Paisley, USA), Streptomycin (100 µg/ml, Invitrogen), L-glutamine (2 mM, Invitrogen), 2-mercaptoethanol (50 µM, Sigma-Aldrich) and 10% heat-inactivated FCS (Invitrogen).

3.4. *In vitro* antigen presentation assay

Freshly isolated mouse spleen DCs ($2x10^3$ cells/well) were co-cultured for 24 hours at 37ºC with the 5/4E8 T cell hybridoma cell line ($2x10^4$ cells/well), specific for the human aggrecan G peptide (11). Wells contained 200 µl culture medium and 1 µg/ml human aggrecan G peptide (ATEGRVRVNSAYQDK) (kindly provided by Szilvia Bosze and Ferenc Hudecz, Research Group of Peptide Chemistry, Hungarian Academy of Sciences, Eötvös Lorand University, Budapest, Hungary). In technical control wells cells were cultured without peptides. 24 hours later, IL-2, produced by the activated 5/4E8 hybridoma cells, was measured by sandwhich ELISA (R&D Systems Inc.). DCs were challenged with different concentrations (0.1 µM-1000 µM) of histamine (Sigma-Aldrich), administered together with the aggrecan peptide. In some experiments, histamine receptor antagonists Famotidine (H_2R ligand, 10 µM, Sigma-Aldrich) and JNJ10191584 (H_4R ligand, 10 µM, Tocris Bioscience, Ellisville, USA) were applied 1 hour before histamine (1 µM) treatment.

Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich) supplemented with Penicillin (50 U/ml), Streptomycin (50 µg/ml), L-glutamine (2 mM) 2-mercaptoethanol (50 µM), 10% heat-inactivated FCS, 1% non-essential amino acids (Sigma-Aldrich) and 1% sodium pyruvate (Sigma-Aldrich) was used in all antigen presentation experiments.

3.5. Migration assay

Migratory response of splenic DCs was examined using 24-well Transwell plates (Corning Incorporated/Costar, New York, USA) with 5 µm-pore-size polycarbonate filters. For an hour before the migration assay, the Transwell system was preincubated with 600 µl and 200 µl medium (complete RPMI-1640) in the lower and the upper chambers, respectively. Then the upper chamber was filled with 200 µl of cell suspension ($1x10^6$ cells/ml) in complete RPMI-1640, and 600 µl of medium with or without histamine (1, 10

and 100 µM) or 4-methylhistamine (4-MH, 1, 10 and 50 µM, Tocris Bioscience) was added to the lower chambers. Migration was allowed for 2 hours at 37 ºC. Transwells were removed, and $2x10^4$ polystyrene microparticles (15 µm in diameter, Fluka Chemie GmbH, Buchs, Sweden) were placed into the lower chambers. The number of transmigrated cells in the bottom chamber was counted by flow cytometry, and was normalized to polystyrene microparticles.

In the experiments using differentiated DCs, 1 µM histamine was added to the cells on days 0, 3rd, 6th and 8th of differentiation.

Migration of 10 days old bone marrow-derived DCs of WT and $H_4R^{-/-}$ was also compared using the above experimental protocol.

3.6. Cell adhesion assay

Adhesion of DCs was monitored in an impedance-based assay using xCELLigence RTCA SP system (Roche Applied Science, Indianapolis, IN, USA) (12). As viable cells are good insulators, the adhesion of cells to small reference electrodes, connected to AC circuits, shows good correlation with the number of attached cells (13). Bottom areas of the wells of E-plates®96 (Acea, San Diego, CA USA) served as gold electrode furnished surfaces for the measurements. Prior to the experiments, the electrodes were coated with 25 µl 0.025% human plasma fibronectin (FC010 Millipore, Temecula, CA, USA) in 0.1% gelatin solution (Sigma-Aldrich) for 20 min at 4 °C. Next, the coating solution was carefully removed from the wells, and 100 µl complete medium (RPMI-1640) was added as a reference substance for baseline measurements. The baseline impedance was detected in a tissue culture incubator (37 °C, 5% CO_2) for 1.5-2 hours. DCs were gently removed, and were suspended in 100 µl culture medium, and seeded into wells (50.000 cells/well in triplicates, n=6). Values of impedance (Z) were recorded in a real-time mode at 10 kHz for 12 hours (sampling frequency of data collection was 1/15 sec). Impedance was represented by the cell index (CI) values $((Z_i - Z_0)$ [Ohm]/15[Ohm]; Z_0: background resistance, Z_i: individual time point resistance) and the delta cell index was calculated for the baseline. Cell Index data were analyzed and the slope of the curve was calculated by RTCA software v1.2 (Roche Applied Science).

3.7. *In vitro* DC stimulation

Spleen-derived CD11c+ DCs were plated in 12-well plates at a density of $3x10^6$ cells/well in complete RPMI-1640 medium. With the exception of the control wells, DCs were incubated in the presence of 1 µg/ml lipopolysaccharide (LPS, Sigma-Aldrich) for 24 hours at 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, Sigma-Aldrich) for 15 min prior to the agonist treatment. After incubation, the cells were processed for RNA isolation and subsequent real-time PCR analysis.

3.8. *In vivo* DC stimulation

WT and $H_4R^{-/-}$ mice (n=6 in each group) were injected intra-peritoneally with either 200 µl CFA emulsion (Sigma-Aldrich) containing 0.1 mg of Mycobacterium tuberculosis H37 Ra 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 quantitative PCR analysis.

3.9. Quantitative real-time PCR

Total RNA was extracted from DCs using RNeasy Mini Kit (Qiagen, Duesseldorf, Germany). After DNase I (Qiagen) digestion, 2 µg total RNA per sample was reverse transcribed using Random Primers (Promega Corp. Madison, WI, USA) and MuLV Reverse Transcriptase (Applied Biosystems, Foster City, CA, USA). Relative quantification of target mRNA was performed with a TaqMan real-time RT-PCR assay on an ABI Prism 7000 Sequence Detection System (Applied Biosystems) following manufacturer's instructions.

Taqman probe sets were used as follows: TNF-alpha, IL-1 beta, IL-6, IL-4, IL-10, Gata-3, IL-12, IFN-gamma, T-bet, CCR7, CCL19 and GAPDH as a housekeeping internal control. All probe sets were purchased from Applied Biosystems. Signal levels normalized to GAPDH were calculated using the comparative C_t method.

3.10. Flow cytometry

Purity of isolated or differentiated DCs was checked by CD11c PE (BD Biosciences PharMingen, San Diego, CA, USA) staining. CD11c+ cells were analyzed by FACS Calibur™ flow cytometer and CellQuest™ software (BD PharMingen).

3.11. Statistics

Statistical analysis was performed using analysis of variance (ANOVA), as appropriate, and Tukey test as post hoc test. A $p<0.05$ was considered significant and was indicated by *, $p<0.01$ was indicated by ** and $p<0.001$ was indicated by ***. The Statistica program version 8 was used for statistical analysis.

4. RESULTS

4.1. Role of histamine and H_4R in DC antigen presentation

It is well established that DCs are highly specialized APCs found in almost all peripheral tissues as well as in primary and secondary lymphoid organs. They have the ability to activate naive T lymphocytes, and thus, play a pivotal role in initiation and regulation of T cell responses. Their migration capacity allows them to circulate in the body and to make contacts with biological mediators such as histamine.

An *in vitro* assay was used to examine the effect of different concentrations of histamine on the antigen-presenting capacity of splenic DCs. The amount of IL-2 produced by 5/4E8 hybridomas was between 4000-10000

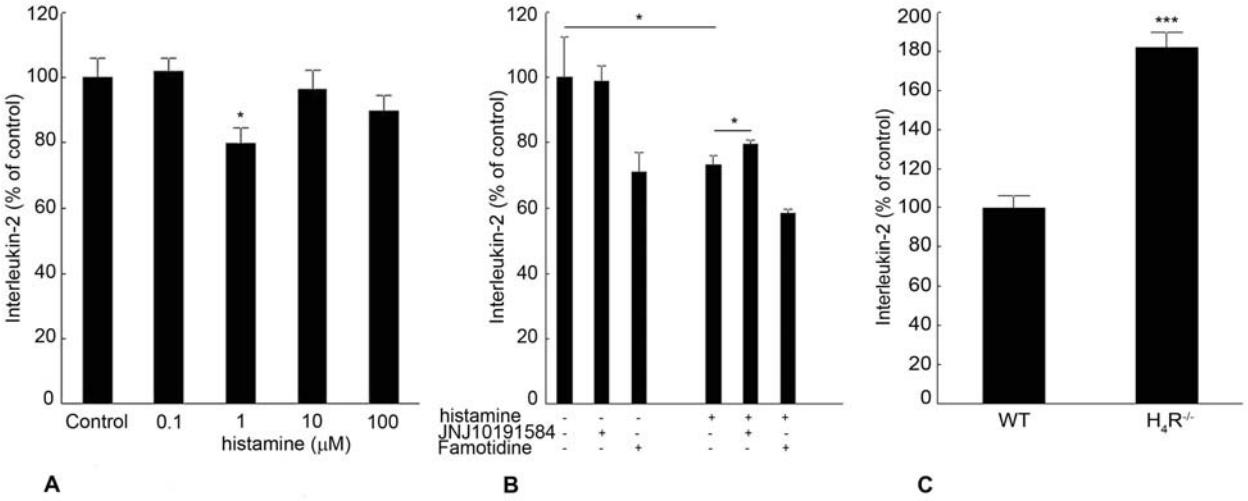

Figure 1. *In vitro* antigen presentation capacity of spleen-derived DCs. (A) Effect of different concentrations of histamine on WT DCs (n = 8). (B) Effect of 1 hour H_2R (Famotidine, 10 μM) and H_4R (JNJ10191584, 10 μM) inhibitors before histamine (HA, 1 μM) treatment on WT DCs (n= 8), (C) WT and $H_4R^{-/-}$ DCs (n = 4). Isolated splenic DCs were cocultured with 5/4E8 T cell hybridoma cells and a human aggrecan G peptide was added as specific antigen. Antigen-specific T cell response was determined by measuring the IL-2 content of supernatants with sandwich ELISA. Values shown are given as means ± SEM percentage of control. Statistical significance was calculated by ANOVA and Tukey HSD, as post hoc test (*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$).

pg/ml in these experiments. The IL-2 production of the peptide-stimulated aggrecan epitope-specific 5/4E8 T cell hybridoma was significantly decreased by histamine (1 μM) showing a concentration-dependent biphasic effect (Figure 1 .A). No IL-2 was detected from those technical control wells, to which no antigen was added. These controls were not presented in figures.

In the following experiments, antigen presentation assay was performed with DCs from WT mice in the presence of H_2R or H_4R antagonists (Famotidine and JNJ10191584, respectively). We found Famotidine to be effective in reduction of antigen presentation capacity, while H_4R antagonist had no influence. When challenging DCs by JNJ10191584 treatment prior to histamine addition, the declined antigen-specific T cell stimulation response to histamine increased marginally ($p = 0.057$). While Famotidine did not antagonize the action of histamine, the H_2R antagonist pretreatment further decreased the effect of histamine, and the untreated control level of antigen-specific IL-2 production was not recovered (Figure 1B).

The amount of IL-2 synthesized by the human aggrecan-specific 5/4E8 T cell hybridoma was significantly higher ($p < 0.001$) when the aggrecan peptide was presented by spleen-derived DCs of $H_4R^{-/-}$ mice compared to WT ones (Figure 1C). Thus, DCs from mice lacking H_4R possess a significantly higher antigen presenting capacity.

4.2. Migration studies

Migration of splenic DCs toward histamine and 4-MH was investigated in a Transwell system. Neither different concentrations (1, 10, 100 μM) of histamine nor the H_4R agonist 4-MH (1, 10, 50 μM) influenced DC migration (Figure 2.A and B).

We hypothesized that a prolonged histamine treatment, if applied during DC differentiation, may cause changes in the migration capacity of the cells. In order to investigate this question, DCs were differentiated in the presence or absence of 1 μM histamine added on days 0, and 3rd; 6th and 8th or 0, 3rd, 6th and 8th of differentiation. On the 10th day, migration of DCs was studied in Transwell system. Figure 2C shows that DC migration was stimulated by histamine in a time-dependent manner, although the increase was not significant.

On the 10th day of bone marrow-derived DC differentiation, migration of WT and $H_4R^{-/-}$ DCs was also compared using Transwell chambers. Figure 3. indicates that DCs derived from H_4R-deficient mice show significantly lower migration capacity than their WT counterparts.

4.3. Adhesion studies

Time course characteristics of DC adhesion are shown in Figure 4.A. After plating, the cells attach and spread relatively fast, which is detected as a steep increase of Cell Index (CI) values. However, the cells of different genotypes have similar adhesion characteristics, in the first period (72 min) the average CI of $H_4R^{-/-}$ cells show a faster increase than that of the WT cells. Five hours after seeding the difference of the two cell types is diminished. The fast phase (72 min) of cell adhesion is also characterized by the slopes of the curves, and shows significant difference of the two investigated DCs; the $H_4R^{-/-}$ curve has increased CI value (0.234 ± 0.028) (Figure 4.B). About 120 min after seeding, the CI shows significantly different values in WT and $H_4R^{-/-}$ cells (0.189± 0.013 and 0.257± 0.015, respectively) (Figure 4.C).

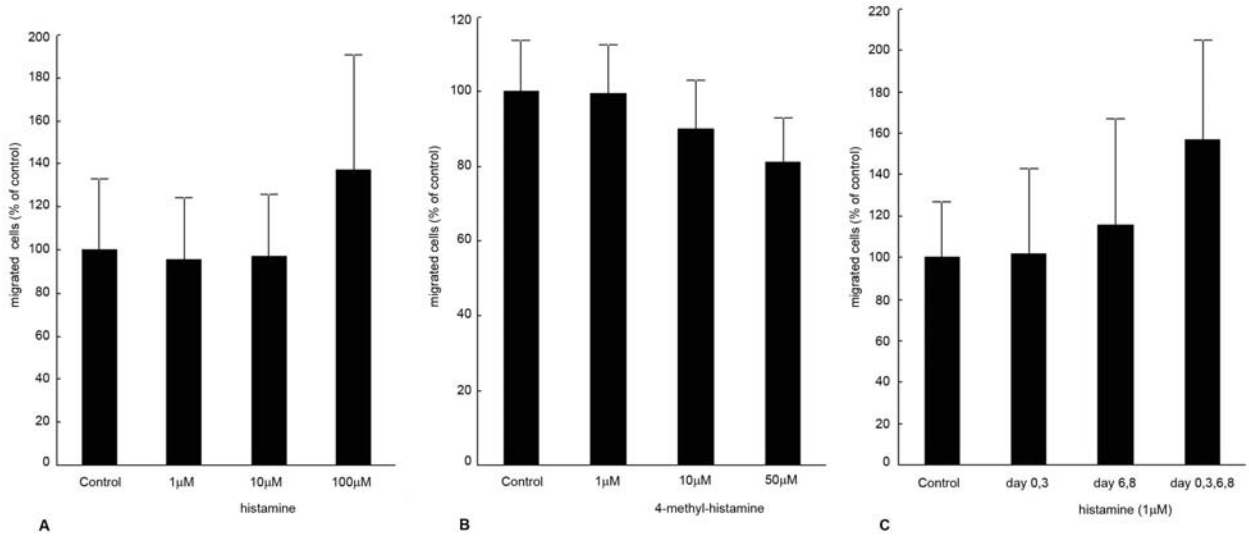

Figure 2. Migration capacity of WT splenic DCs challenged by different concentrations of (A) histamine (n = 6) and (B) a H_4R agonist, 4MH (n = 12). (C) Migration capacity of WT bone marrow-derived differentiated DCs, treated with 1 μM histamine during differentiation, was indicated under the column (n = 5). The number of DCs that migrated through the Transwell membrane, was measured by flow cytometry and the numbers were normalized to $2x10^4$ microparticles. Values shown as means ± SEM percentage of control. Statistical analysis was performed using one-way ANOVA, and Tukey as post hoc test.

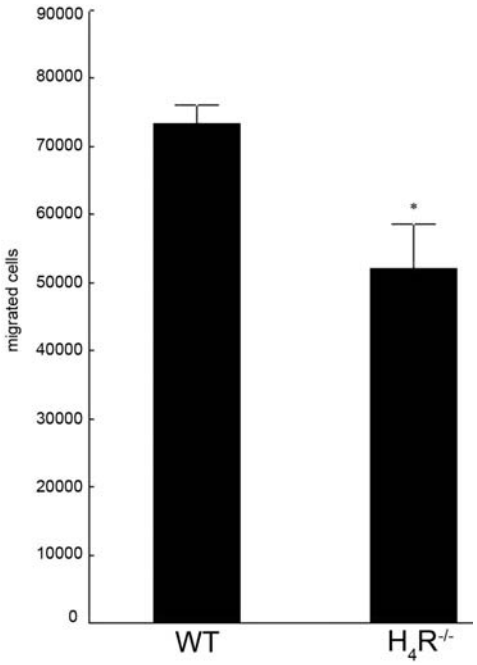

Figure 3. Migration capacity of bone marrow-derived differentiated DCs of WT and $H_4R^{-/-}$ mice. The number of DCs that migrated through the Transwell membrane, was measured by flow cytometer and were normalized to $2x10^4$ microparticles. Values shown are means ± SEM of 7 independent experiments. Statistical analysis was performed using one-way ANOVA, and Tukey as post hoc test. (*: $p<0.05$)

4.4. Cytokine production of *in vitro* stimulated DCs

One representative feature of DCs is their ability to activate naive T lymphocytes in an antigen-dependent manner. DCs play a role in both T cell polarization and regulation of the intensity of the adaptive immune response. Cytokines and chemokines, produced by DCs, play essential role in these processes. Therefore, in an *in vitro* system we investigated cytokine production of splenic DCs following 15 min H_4R agonist, 4MH (0.1 μM) and 24 hours LPS (1 μg/ml) treatment. In order to clarify the role of H_4R, we used specific antagonist, JNJ7777120 (10 μM), added to cells 15 min before agonist challenge. We found

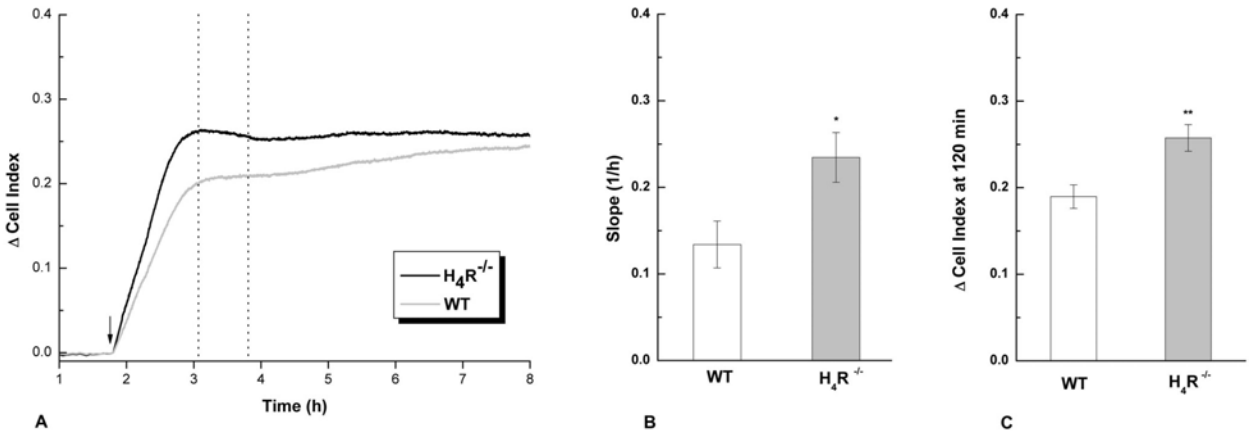

Figure 4. Attachment and spreading of WT and $H_4R^{-/-}$ DCs on microelectrode sensors were monitored by a real-time cell electronic sensing system. The Δ Cell Index was calculated for the baseline at the time of the cell seeding (↓) and represent the average value of 6 parallel experiments in triplicate. (A) Slopes of the curves were analysed for the first 72 mins (----) and ΔCell Index data were obtained after 120 mins (----) of seeding. (B) Average of the slope values of the cell index curve for the first 72 minutes (n = 6). (C) Average of delta cell index data at 120 minutes after seeding (n = 6).

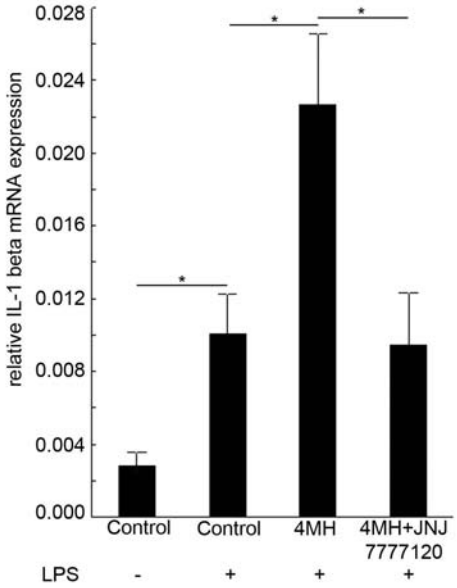

Figure 5. Cytokine response of *in vitro* stimulated WT DCs. DCs were treated with a H_4R agonist, 4MH (0.1 μM) for 15 minutes alone or along with a H_4R antagonist, JNJ7777120 (10 μM) for 15 minutes prior to agonist challenge, followed by LPS (1 μg/ml) treatment. After 24 hours the expression level of IL-1 beta was measured by real-time PCR. Values shown are means ± SEM of 4 independent experiments. Statistical significance was calculated using one-way ANOVA, and Tukey as post hoc test. (*: $p<0.05$).

that mRNA expression of IL-1 beta, one of the paramount inflammatory cytokines, was elevated after a combined treatment with a H_4R agonist and LPS. JNJ7777120, a H_4R antagonist, reversed this effect significantly (Figure 5).

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

WT and $H_4R^{-/-}$ mice (n = 6 in both groups) were injected with CFA. We monitored the expression of various cytokines, chemokines and major transcription factors involved in Th1, Th2 responses and Treg function of isolated splenic DCs by real-time PCR. We detected significantly lower IL-10, Gata-3, IL-12 and IFN-gamma mRNA expression in $H_4R^{-/-}$ DCs cells compared to WT controls. Among the genes tested, CFA treatment reduced the expression of IL-1 beta, IL-4, IL-10, CCL19 and CCR7 significantly, regardless of genotype of DCs. We also found genotype-dependent differences in the response to CFA; while CFA treatment reduced IL-6, Gata-3, and T-bet expression in WT animals, it was uneffective in H_4R-

deficient DCs. In KO mice IFN-gamma mRNA level was elevated by the *in vivo* CFA injection. In contrast, it was not altered in WT animals (Figure 6 A-K).

5. DISCUSSION

DCs are considered as main antigen presenting cells of mammals. They have the capacity to present foreign antigens very efficiently to naive T lymphocytes, and to stimulate them specifically. With the activation of T cells, DCs serve as a link between innate and acquired immune systems, so they can be regarded as conductors of immune regulation.

Antigen presentation can be studied both *in vivo* and *in vitro*. We have applied a sensitive system that we developed and described earlier (11, 14). In this *in vitro* assay the IL-2 protein production of a human aggrecan-specific 5/4E8 T cell hybridoma cell line was induced by splenic DCs loaded by a human aggrecan peptide.

DCs, circulating from bone marrow to the periphery and to secondary immune organs, may come into contact with histamine secreted by mast cells or basophils. Furthermore, DCs themselves have been also shown (2, 3) to produce histamine. However, up till now only few data demonstrate the influence of histamine on antigen presenting capacity of DCs. We have shown earlier that DCs from histamine-free mice ($HDC^{-/-}$) are characterized by an increased antigen presentation capacity compared to WT ones (14). It is well documented that in the immunological synapse, at the interface between DCs and T cells, interactions of T cell receptors with MHC-peptide complexes, as well as those between costimulatory molecules are necessary. The higher expression of the CD86 costimulatory molecule in human DCs (15) and the increase of MHCII protein in murine DCs (16) have been demonstrated in response to histamine treatment. Histamine has been reported recently to stimulate *in vitro* CD8+ T cell-mediated cross-presentation, although the expression of MHCI, a molecule that plays a significant role in cross-presentation, was also found to be reduced in this system (16).

In our experiments we detected a significantly decreased antigen presentation capacity of murine splenic DCs in the presence of histamine (1 μM). The response was concentration-dependent biphasic. Biphasic (U-shaped) dose-response curve is not an usual, but is not so rare relationship. It has been published that e.g. histamine evoked concentration-dependent biphasic effect on human bronchial arteries (17). It is possible that in the case of ligands like histamine having multiple receptors and signalling pathways simultanously, different doses are needed for the activation of certain pathway.

Previously we have characterized the expression of H_4R in spleen-derived murine DCs (18). In order to study the possible involvement of H_4R in antigen presentation, we compared this activity of WT and $H_4R^{-/-}$ mouse-derived DCs. Significantly higher amount of IL-2 was produced when the 5/4E8 T cell hybridoma cells were stimulated by peptide-loaded DCs from H_4R-deficient mice as compared to DCs from WT controls.

To gain more insight into the role of histamine in antigen presentation, we used Famotidine, a H_2R antagonist and JNJ1091584, a H_4R antagonist prior to histamine treatment of DCs from WT mice. Surprisingly, regardless of the presence or absence of histamine, Famotidine reduced antigen presentation. It might be hypothesized that this effect, exhibited without histamine, was non specific. On the other hand, JNJ10191584, a H_4R antagonist, when applied alone, did not alter the antigen-specific T cell response. In contrast, the histamine-induced reduction was almost completely reversed, suggesting the possible implication of H_4R in histamine effect. This observation is supported by our former results that showed a declined antigen presentation in response to the treatment by 4MH, a H_4R agonist (18).

At the same time, based on our data, it should be emphasized that long-term H_4R deficiency had more pronounced impact on antigen presentation than the short-term antagonist treatment. The molecular mechanism by which histamine acts on antigen presentation via H_4R, remains unknown. We could not detect any difference in either MHCII or costimulatory molecule expression of histamine-treated H_4R-KO and WT splenic DCs (data not shown). On the other hand, IFN-gamma has been shown to affect certain components of the antigen presentation machinery (19, 20). Since we found that the induction of IFN-gamma production was more pronounced in H_4R-KO DCs compared to WT ones, the enhanced IFN-gamma level may account for the elevated T cell-stimulatory capacity of H_4R-deficient DCs.

DCs are characterized by high migration capacity exerting their functions at different locations of the body, from the blood through peripheral tissues to lymphoid organs. On their route DCs are under the influence of various chemical factors including chemokines, cytokines or smaller molecules like histamine. Numerous compounds may moderate the motility of DCs through paracrine or autocrine mechanisms. Several studies documented the role of histamine and H_4R in the chemotaxis of different immune cells such as eosinophils (21), mast cells (22) and natural killer cells (23). Regarding DCs of human origin, the role of H_2R and H_4R have been implicated in this process (8). In mouse DCs, differentiated from bone marrow, histamine (1 mmol/l) was suggested to be involved in the regulation of migration via H_1R and H_4R (24). It has to be added at the same time that in the majority of studies histamine and its different antagonists were applied in much lower concentrations than 1 mmol/l.

In our migration experiments first we tested migratory properties of splenic DCs challenged with different concentrations of histamine and 4MH, a H_4R agonist, using an *in vitro* Transwell system. The migration capacity of splenic DCs was not changed significantly by either histamine or 4MH. It is well established that the chemokine, CCL19 and its receptor, CCR7 play a pivotal role in DC trafficing (25). We could not detect any difference in the mRNA expression between WT and H_4R-

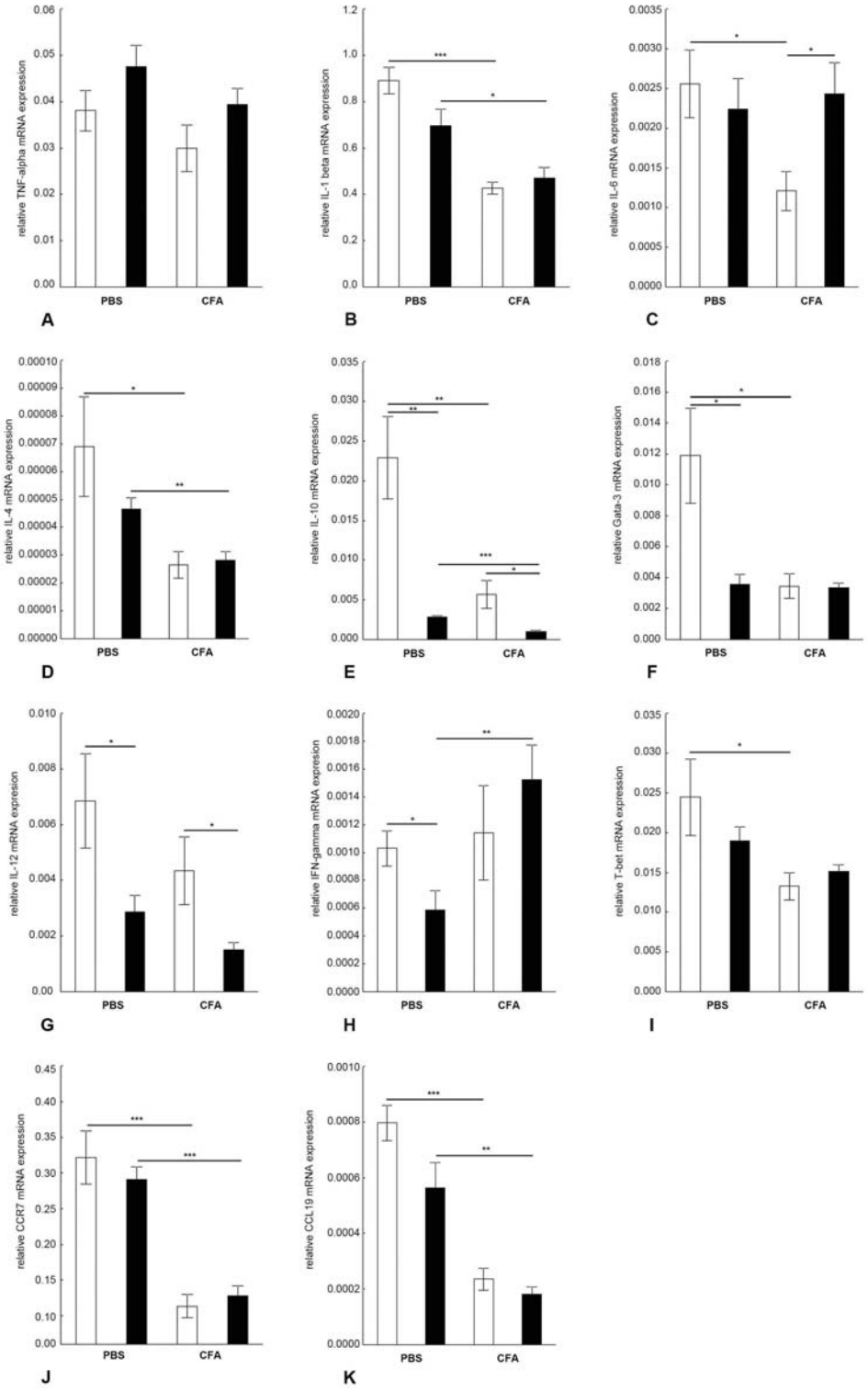

Figure 6. Cytokine responses of *in vivo* activated WT (□) and $H_4R^{-/-}$ (■) DCs. WT and $H_4R^{-/-}$ mice were injected CFA containing stable emulsion (200 µl/mouse intra-peritoneally). After 7 days the TNF-alpha, IL-1 beta, IL-6, IL-4, IL-10, Gata-3, IL-12, IFN-gamma, T-bet, CCR7, CCL19 expressions of isolated splenic DCs were measured by real-time PCR. Values are shown as means ± SEM of 6 independent experiments. Statistical significance was calculated using two-way ANOVA, and Tukey as post hoc test. (*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$).

KO spleen-derived DCs. Hypothesizing that splenic DCs are less sensitive to short-term stimulus than freshly differentiated ones, we examined long-term histamine stimulation during DC differentiation. Different length histamine treatment was used during the ten days differentiation period. Histamine was added to DCs on days 0 and 3, 6 and 8 or on days 0, 3, 6 and 8 of *in vitro* differentiation. Migration assay was performed on the 10th day. Although our results were not significant, the number of transmigrated cells increased in correlation with the length of histamine treatment.

Recently, H_4R has been shown to direct the migration of different immune cells including eosinophils, mast cells and DCs. Thus, we compared migration capability of WT and $H_4R^{-/-}$ DCs differentiated from bone marrow. We experienced that DCs having defective H_4R, possessed significantly lower migration capacity than their WT counterparts suggesting the necessity of H_4R in the regulation of this process.

We were the first to use an impedance-based xCELLigence RTCA SP system to study the adhesion of murine differentiated DCs. We found that both parameters characterizing adhesion of the cells, slope and ΔCell index, were significantly higher in H_4R-KO cells than in WTs. For the first sight these results were surprising since previously we detected impaired migration capacity of H_4R-deficient DCs. Adhesion is a prerequisite of migration, so the above processes were expected to show direct correlation. But recently, it has been published that murine DCs migration could be negatively regulated by adhesion (26). Evidently, further experiments are required to obtain more precise molecular data about the action of histamine via H_4R governing adhesion and migratory processes.

DCs due to their potent capacity to activate naive T lymphocytes, as well as to their role in the modulation of immune responses, are crucial components of the immune system. Evidences 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. Of course, cytokines and chemokines, produced by DCs, have a large impact on this regulatory effect, too. The pro-inflammatory cytokine IL-1 beta is a crucial mediator of the innate immune system. Beside activated mononuclear phagocyte cells, DCs are also important producers of this molecule. Mazzoni *et al*. have shown that histamine blocked LPS-induced IL-1 beta expression in immature human DCs (1). In our work we also tested IL-1 beta mRNA expression using either H_4R agonist or antagonist treatment followed by LPS stimulation. Real-time PCR results revealed that the LPS-induced expression of IL-1 beta was further induced by the H_4R agonist 4MH, while the H_4R neutral antagonist JNJ7777120 effectively reversed this effect demonstrating the participation of histamine via H_4R in governing IL-1 beta expression.

It has been long known that DC-derived cytokines have an impact on Th1/Th2 balance and the outcome of immune response. There is also evidence that histamine favors the induction of Th2-biased responses by altering the cytokine profile of DCs (4). Before the discovery of H_4R, mainly H_2R was suspected to play a role in DC cytokine production (1, 9). Earlier results need to be reassessed in order to establish H_4R activity in this field. Most of the data concerning 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. DCs were separated from spleens of WT and H_4R-deficient animals 7 days after CFA injection. We have demonstrated earlier that DCs, differentiated *in vivo* in the absence of histamine ($HDC^{-/-}$ DCs), exhibit Th1-type cytokine dominance upon CFA stimulation (14).

We observed significantly lower mRNA expression of both Th2 and Th1 response-related molecules in $H_4R^{-/-}$ DCs. Most authors found opposite *in vitro* action of histamine on Th1 and Th2 type cytokine expression, detecting augmentation of Th2 and reduction of Th1 molecules, and these authors demonstrated H_2R transmission (27, 9). Gutzmer *et al.* reported suppressed IL-12p70 production and no change in IL-10 secretion upon the addition of histamine to human monocyte-derived DCs. The effect was found to be mediated by both H_2 and H_4 receptors (8).

We could not measure any changes in the expression of TNF-alpha, which result was similar to *in vitro* findings of others (3). In some cases genotype-dependent CFA effect was found. CFA challenge decreased IL-6, Gata-3 and T-bet expression in WT DCs, while in H_4R-KO mice it was found to be uneffective. Regarding IL-6 cytokine our real-time PCR result contradicts the observation of Dunford *et al*. In *in vitro* conditions using different stimulations, they detected declined IL-6 protein in DCs having malfunctional H_4R. In the expression of IFN-gamma another sort of effect was observed, namely CFA stimulated $H_4R^{-/-}$ DCs produced more IFN-gamma, on the other hand there was no change in IFN-gamma mRNA level of WT aminals. Previously we have published similar difference between IFN-gamma expression of WT and $HDC^{-/-}$ DCs that strongly suggests the involvement of H_4R in the regulation of this cytokine synthesis. Taken together, the H_4R-mediated histamine modulation of Th1, Th2 or Treg-related gene expression seems to be cytokine dependent.

In summary, we demonstrated the impact of histamine mediation through H_4R in several cellular activities of murine DCs. The absence of H_4R resulted in an enhanced antigen presentation capability and adhesion characteristics. Furthermore, H_4R deficiency decreased 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 in an indirect manner. Naturally, further investigations are required to elucidate the precise molecular mechanisms of H_4R-mediated histamine activity in various DC mechanisms.

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Abbreviations: Dendritic cell: DC, Histamine H4 receptor: H_4R, Antigen presenting cell: APC, 4-methylhistamine: 4-MH, lipopolysaccharide: LPS

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