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RESEARCH PAPER

Antagonism of histamine H_4 receptors exacerbates clinical and pathological signs of experimental autoimmune encephalomyelitis

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BACKGROUND AND PURPOSE

The histamine H_4 receptor has a primary role in inflammatory functions, making it an attractive target for the treatment of asthma and refractory inflammation. These observations suggested a facilitating action on autoimmune diseases. Here we have assessed the role of H_4 receptors in experimental autoimmune encephalomyelitis (EAE) a model of multiple sclerosis (MS).

EXPERIMENTAL APPROACH

We induced EAE with myelin oligodendrocyte glycoprotein ($MOG_{35–55}$) in C57BL/6 female mice as a model of MS. The histamine H_4 receptor antagonist 5-chloro-2-[(4-methylpiperazin-1-yl)carbonyl]-1*H*-indole (JNJ7777120) was injected i.p. daily starting at day 10 post-immunization (D10 p.i.). Disease severity was monitored by clinical and histopathological evaluation of inflammatory cells infiltrating into the spinal cord, anti-$MOG_{35–55}$ antibody production, assay of T-cell proliferation by [^{3}H]-thymidine incorporation, mononucleate cell phenotype by flow cytometry, cytokine production by ELISA assay and transcription factor quantification of mRNA expression.

KEY RESULTS

Treatment with JNJ7777120 exacerbated EAE, increased inflammation and demyelination in the spinal cord of EAE mice and increased IFN-γ expression in lymph nodes, whereas it suppressed IL-4 and IL-10, and augmented expression of the transcription factors Tbet, FOXP3 and IL-17 mRNA in lymphocytes. JNJ7777120 did not affect proliferation of anti-$MOG_{35–55}$ T-cells, anti-$MOG_{35–55}$ antibody production or mononucleate cell phenotype.

CONCLUSIONS AND IMPLICATIONS

H_4 receptor blockade was detrimental in EAE. Given the interest in the development of H_4 receptor antagonists as anti-inflammatory compounds, it is important to understand the role of H_4 receptors in immune diseases to anticipate clinical benefits and also predict possible detrimental effects.

LINKED ARTICLES

This article is part of a themed issue on Histamine Pharmacology Update. To view the other articles in this issue visit http://dx.doi.org/10.1111/bph.2013.170.issue-1

Abbreviations

EAE, experimental autoimmune encephalomyelitis; GFAP, glial fibrillary acidic protein; H&E, haematoxylin and eosin; LFB, Luxol fast blue; LN, lymph nodes; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis; NeuN, neuronal specific nuclear protein; PHA, phytohaemaglutinin.

Introduction

Multiple sclerosis (MS) is the most common, non-traumatic cause of neurological disability among young adults in Western Europe and North America. Experimental autoimmune encephalomyelitis (EAE) has been extensively used as a murine model of MS as both diseases are characterized by CNS inflammation with focal lymphocytic infiltrations that lead to damage of myelin and axons associated with neurological dysfunction. In recent years, the histaminergic system has been postulated to have a role in the pathogenesis of autoimmune diseases (Ma *et al.*, 2002), and there are several lines of evidence suggesting a key regulatory role of histamine in EAE (Musio *et al.*, 2006). Therefore, strategies aimed at interfering with the histamine axis may have relevance in the therapy of autoimmune diseases of the CNS. The regulatory functions of histamine relevant to the onset and progression of neuroinflammatory diseases, particularly EAE, are being studied in genetically modified mice lacking histamine receptors and with selective histamine agonists and antagonists. Preclinical studies on EAE have shown that histamine plays a complex role with varying and opposite effects, depending on the receptor subtypes being activated and the targeted tissue (see Passani and Ballerini, 2012; receptor nomenclature follows Alexander *et al.*, 2011). All histamine receptors are expressed on the complement of cells involved in autoimmune diseases, with the exception of the H_3 receptor that is normally not expressed by haematopoietic cells, but is mostly confined to the CNS (Passani and Blandina, 2011). H_1 and H_2 receptors seem to have a proinflammatory role and disease-promoting effect. Susceptibility to EAE requires expression of *Hrh1*, the gene encoding the H_1 receptor (Ma *et al.*, 2002). H_1/H_2 receptor transcripts are present in the brain lesions of mice with active EAE, and administration of pyrilamine, a H_1 receptor antagonist, reduces EAE severity (Pedotti *et al.*, 2003). On the other hand, deletion of the H_3 receptor leads to more severe EAE, an effect associated with altered blood–brain barrier permeability (Teuscher *et al.*, 2007). The majority of cells expressing histamine H_4 receptors are haematopoietic in lineage (see Zampeli and Tiligada, 2009), including the complement of cells involved in autoimmune diseases (Mommert *et al.*, 2011).

The aim of our study was to assess the effect of H_4 receptor pharmacological blockade on several EAE parameters. The identification of JNJ7777120 as a potent and selective H_4 receptor antagonist (Thurmond *et al.*, 2008) made it possible to elucidate some of the roles of this receptor in a variety of allergic and inflammatory processes. Functions of the H_4 receptor include mediation of calcium mobilization, shape change, actin polymerization (Barnard *et al.*, 2008) and chemotaxis of mast cells and eosinophils (Gutzmer *et al.*, 2005), up-regulation of adhesion molecules (Buckland *et al.*, 2003), release of $CD8^+$T lymphocytes (Amaral *et al.*, 2010) and suppression of IL-12p70 production in dendritic cells (Gutzmer *et al.*, 2005). Expression of H_4 receptors is dynamic as it is up-regulated during the differentiation from human monocytes to dendritic cells (Gutzmer *et al.*, 2005). In addition, receptor levels change with the progression of the pathophysiological response, for example, inflammatory stimuli can up-regulate the expression of H_4R in monocytes (Dijkstra *et al.*, 2007). Interestingly, in a human autoimmune salivary gland disease (Sjögren's syndrome), there is down-regulation of H_4 receptors expressed on acinar cells (Stegaev *et al.*, 2012). Also, H_4 receptor activation may control inflammation by suppressing the production of cytokines, as well as chemokines in antigen-presenting cells (APCs) and thereby can contribute to the shift from a Th1- to a more Th2-dominated profile (Morgan *et al.*, 2007). Furthermore, pilot data revealed that H_4 receptors are expressed on the soma of both Aδ and C-fibre sensory neurons with intense staining of small and medium diameter neurones as well as lamina I-III of the rat lumbar spinal cord, where the immunoreactivity pattern suggests localization with terminals of primary afferent neurons (Lethbridge and Chazot, 2010; Katebe *et al.*, 2012). Supposedly, H_4 receptor antagonists may relieve itch by decreasing not only inflammation but also the urge to scratch. Therefore, despite the uncertainties regarding the mechanisms of action, the scientific community is displaying great interest in the therapeutic potential of H_4 receptor antagonists. Over the past decade, H_4 receptor antagonists have been suggested as potential drug candidates for the treatment of refractory inflammatory and immune diseases. Surprisingly, H_4 receptor-knockout (H_4R-KO) mice develop a more severe $(MOG)_{35–55}$-induced EAE compared to wild-type mice (del Rio *et al.*, 2012). As genetically modified mice may carry alterations of systems other than the targeted ones, and activation of vicarious mechanisms may hinder the effects related to the deleted gene, we studied myelin oligodendrocyte glycoprotein $(MOG)_{35–55}$-induced EAE in mice treated daily with JNJ7777120. Consistent with the results obtained in H_4R-KO mice, pharmacological blockade of H_4 receptors with JNJ7777120 worsened EAE clinical and histopathological parameters. Given the interest in the development of H_4 receptor antagonists as anti-inflammatory compounds, we believe that our results will help anticipate clinical benefits or predict possible detrimental effects in the treatment of autoimmune diseases and in MS, in particular.

Methods

Experimental animals

All animal care and experimental procedures were performed according to the European Community guidelines for animal care (DL 116/92, application of the European Communities Council Directive 86/609/EEC) and approved by the Committee for Animal Care and Experimental Use of the Universita' di Firenze (I). All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (McGrath *et al.*, 2010). A total of 38 female C57BL/6 mice were used for immunization experiments. Mice were randomly assigned to standard cages, with four to five animals per cage, and kept at standard housing conditions with a light/dark cycle of 12 h (08.00–20.00 hours) and free access to food and water.

Induction and clinical assessment of EAE

C57Bl/6 female mice, 6–8 weeks of age, were obtained from Harlan Italy Srl. (Milan, Italy). The mice were housed in macrolon cages on a 12 h light/dark cycle at room temperature (23°C), with *ad libitum* access to food and water.

Adequate measures were taken to minimize pain or discomfort. Mice were immunized s.c. in the flanks and at the base of the tail with a total of 200 μg of $MOG_{35–55}$ (synthesized by EspiKem Srl., Universita' di Firenze, Italy) per animal emulsified in complete Freund's adjuvant (Sigma, Milan, Italy) supplemented with 4 mg·mL^{-1} of *Mycobacterium tuberculosis* (strain H37Ra; Difco Laboratories, Detroit, MI, USA). Immediately thereafter, and again 48 h later, the mice received an i.p. injection of 500 ng *Pertussis* toxin (Sigma) in 100 μL of PBS. The animals were examined daily for weight loss and disability, and were clinically graded by investigators, unaware of groups treatments, as follows: 0 indicates no signs; 0.5, partial loss of tail tonicity; 1, paralysed tail; 2, ataxia and difficulty in righting; 3, paralysis of the hind limbs and/or paresis of the forelimbs; 4, tetraparalysis; 5, moribund or death.

Pharmacological treatments

The H_4 receptor antagonist JNJ7777120 (Johnson &Johnson, San Diego, CA, USA) was dissolved in 1% ethanol in physiological saline to give a final dose of 10 mg·kg^{-1} JNJ7777120 or vehicle in 100 μL per mouse, and administration daily i.p. injections for the entire duration of the experiment (up to 28 days after immunization). Mice were randomly assigned to two different experimental groups: mice that received daily injections of either JNJ7777120 or vehicle beginning at D10 p.i. and were killed at D28 p.i., and mice that received daily injections of either JNJ7777120 or vehicle beginning at D10 p.i. and were killed at D18 p.i.

Neuropathological evaluations

At the time of killing, the mice were anaesthetized with pentobarbital (65 mg·kg^{-1}, i.p.). The spinal cord was removed from the column and fixed in 4% (v/v) paraformaldehyde in PBS and subsequently paraffin-embedded. Transverse sections (5μm thick) were cut and placed on glass slides. Serial sections were stained with haematoxylin and eosin (H&E) or Luxol fast blue (LFB)-cresyl violet.

Immunohistochemistry

Sections were subjected to antigen retrieval by microwave incubation in 10 mM Na–citrate buffer (pH 6.0) and subsequently immunostained. Briefly, sections were incubated overnight at 4°C with the primary antibody at the optimized working dilution prepared in 0.1 M PBS (pH 7.4) with Triton X-100 (0.3%) and BSA (5 mg·mL^{-1}). The following primary antibodies were used: anti-neuronal specific nuclear protein (NeuN; 1:1000 dilution, Chemicon International, Temecula, CA, USA) to visualize neurons, anti-glial fibrillary acidic protein (GFAP; 1:500 dilution, DakoCytomation, Glostrup, Denmark) to detect astrocytes, Iba1 (1:100 dilution, Wako Chemicals, Neuss, Germany) to detect microglia; anti-IFNγ (1:100 dilution, BioLegend, Aachen, Germany). On the second day, the sections were incubated for 1 h with the secondary antibody prepared in 0.1 M PBS plus BSA (1 mg·mL^{-1}) and immunostaining was visualized with antibodies conjugated with Cy3 (Jackson ImmunoResearch, Suffolk, UK) and Alexafluor 488 (Molecular Probes, Eugene, OR, USA). Sections were coverslipped in Vectastain fluoromount with DAPI (Vector Laboratories, Burlingame, CA, USA). An Olympus BX40 microscope coupled to analySIS^B Imaging Software (Olympus, Milan, Italy) was used to acquire representative images.

Cells

Cells were isolated from lymph nodes (LNs), spleen and spinal cord, and analysed for proliferative response and phenotype as previously described (Gourdain *et al.*, 2012). Briefly, lymphocytes from $MOG_{35–55}$-immunized mice were cultured in complete RPMI in 96-well plates (2×10^5 cells per well) and stimulated with antigen. At day 3, the proliferative response was measured by [^{3}H]-thymidine incorporation test as described previously (Luccarini *et al.*, 2008). To determine mononuclear cell phenotype, surface markers were evaluated by anti-mouse conjugated monoclonal antibodies [CD4 (AlexaFluor 488), IFN-γ (PerCP-Cy5.5)], anti-rabbit H_4 receptor antibodies (Lethbridge and Chazot, 2010), revealed with anti-rabbit Alexafluor-488 secondary antibodies/Cy3 secondary antibodies, CD11c (PerCpCy5), CD11b(PE), CD3 (PE) from AbCam (Cambridge, UK), BioLegend and eBioscience (both San Diego, CA, USA) respectively]. Cells were analysed on a four-colour Epics XL cytometer (Expo32 software; Beckman Coulter, Milano, Italy). Cell viability was tested by means of propidium iodide or 7-actinomycin staining (Molecular Probes). Briefly, staining was performed in PBS 1% FCS for 25 min at 4°C followed by two washes. Later on, cells were analysed by flow cytometry. Invariant natural killer T (iNKT) cells were isolated from the spleen of EAE mice by means of NK1.1$^+$ iNKT Cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany).

Determination of serum antibodies by ELISA

Serum was obtained from three animals in each treatment group killed at D28 p.i., according to standard procedures (Gourdain *et al.*, 2012). Five serum dilutions ranging from 1:50 to 1:1600 were distributed on polystyrene ELISA plates pre-coated with $MOG_{35–55}$ peptide in order to obtain a titration curve. In the same plates we seeded serum from non-immunized mice matched for age and gender as control samples. Bound antibodies were revealed with goat anti-mouse IgM + IgG + IgA (H + L) immunoglobulin conjugated to alkaline phosphatase (Southern Biotech, Birmingham, AL, USA) and *p*-nitrophenyl phosphate as substrate. Absorbance was measured at 405 nm. Results are reported as OD; mean OD values from control samples plus 2 SD were taken as the cut-off value. In our test, 0.02 OD was the lower limit of sensitivity, for the presence of specific antibodies against $MOG_{35–55}$ peptide.

Cytokine determination

Cells isolated from draining LNs at D18 p.i. were cultured at 1×10^6 cells per well in a 48-well plate with phytohaemagglutinin (PHA, 5 μg·mL^{-1}, Sigma-Aldrich) or $MOG_{35–55}$ (2 μg·mL^{-1}, EspiKem Srl.); supernatants were collected after 48 h and evaluated by ELISA for cytokine contents (IFN-γ, IL-6 and IL-10). For IL-4 determination, samples were collected after 5 days. All ELISAs were performed with ELISA Ready-SET-Go! (eBioscience, San Diego, CA, USA).

RNA extraction and quantitative real-time reverse transcription (RT)-PCR

Total RNA was extracted from LN cells isolated at D18 p.i. using Qiazol following manufacturer's protocol (Qiagen, Hilden, Germany). Each mRNA sample was measured for quantification by NanoDrop (Celbio, Milan, Italy). cDNA was then synthesized with RT Quantitect (Qiagen). Real-time PCR was performed with an ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Monza, Italy), according to the manufacturer's instructions. All PCR amplifications were performed by MicroAmp optical 96-well reaction plate with TaqMan Universal Master Mix and with Assay-on-Demand (Applied Biosystems). Each assay was carried out in duplicate and included a no-template sample as negative control. RT-negative samples were used to demonstrate that the signals obtained were RT-dependent. Relative expression of mRNA levels was determined by comparing experimental levels with a standard curve generated with serial dilution of cDNA obtained from human PBMCs. Ubiquitin carboxy-terminal hydrolase L1 (HPRT-1, hypoxanthine phosphoribosyltransferase 1) was used as a housekeeping gene for normalization. In each sample the level of the following mRNA were evaluated: GATA3, Tbet, FOXP3 and RORc, IL-17A. The ratio of transcription factors was calculated within each animal and means were compared.

Data analysis

Statistical analyses were performed using GraphPad Prism version 5 (San Diego, CA, USA). Mean and SEM were calculated for illustration in figures. For each animal, the onset day was recorded as the day post-immunization (D) when the first clinical manifestations appeared (score > 0). Differences between groups were tested by Mann–Whitney test, for their score course. Significant differences between controls and JNJ7777120 treated-mice were established using the Student's *t*-test for the day of onset and of maximum score. Comparisons of parameters such as cytokines, cell proliferation and phenotypes, MOG antibodies, were performed using the unpaired Student's t-test. In all cases, *P*-values less than 0.05 were considered statistically significant.

Results

Treatment with an H_4 receptor antagonist increases the clinical severity of EAE

For our sub-chronic treatment with the H_4 receptor antagonist JNJ7777120, we selected a dose (10 mg·kg^{-1}. i.p.) that was previously shown to attenuate pruritus (Dunford *et al.*, 2007) and airway inflammation when administered orally to BALB/c mice (Cowden *et al.*, 2010). We started treatments at D10 p.i., approximately when clinical signs become apparent. All mice developed EAE symptoms at 9–12 days following $MOG_{35–55}$ immunization and a maximum disease severity around D18 p.i. Using this dose and method of administration, JNJ7777120 was assessed for its effect on the clinical course of EAE. As shown in Figure 1, treatment significantly increased the severity of EAE, compared with vehicle-treated mice. JNJ7777120-treated mice exhibited more severe paralysis that

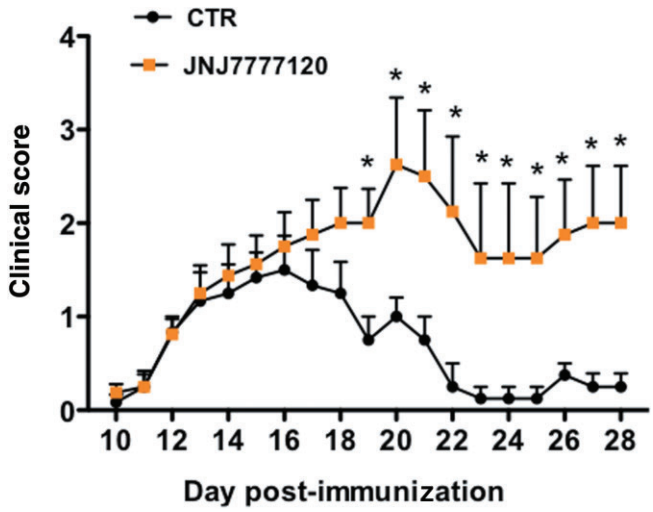

Figure 1

Effect of the H_4 receptor antagonist JNJ7777120 on clinical severity of mice with EAE. C57BL/6 mice were immunized with $MOG_{35–55}$ and treated with 10 mg·kg^{-1} JNJ7777120 daily starting at D10 p.i. until killed (D28 p.i.). Mean clinical scores were increased in JNJ7777120-treated compared with vehicle-treated mice. Shown are group scores means ± SEM of 8–10 mice in three independent experiments. *$P < 0.05$.

became significant at D19. As EAE is thought to be mediated by CD4$^+$ T-cells and APCs, autoreactive immune cells that infiltrate into the CNS, we tried to delineate the peripheral immune mechanism underlying increased EAE severity of JNJ7777120-treated mice. We found that differences in disease score were not associated with differences in T-cell proliferative response against the immunizing antigen $MOG_{35–55}$ (Figure 2A), nor T-cell and APC migration in LNs of EAE mice (Figure 2B). Furthermore, there was no significant difference in H_4 receptor expression on the autoimmune cell subpopulations (Figure 2B). Also, specific $MOG_{33–35}$ antibody titration curves were comparable between vehicle- and JNJ7777120-treated animals, indicating that there were no differences in antibody production in the sera of EAE mice (Figure 2C).

Treatment with a H_4 receptor antagonist increases inflammation and demyelination in the spinal cord of EAE mice

Infiltration of autoreactive immune cells into the CNS results in inflammation of CNS parenchyma and demyelination of motoneurons with consequent paralysis. Following EAE induction, both JNJ7777120- and vehicle-treated mice had distinct areas of immune cell infiltration in the spinal cord as revealed by H&E staining (Figure 3A, left panels). However, JNJ7777120-treated mice had visually more infiltrates than vehicle-treated mice. The degree of myelin loss was visually assessed by LFB staining, which revealed larger plaques of demyelination at the site of inflammatory cell infiltrates in the spinal cord of JNJ7777120-treated mice, compared with vehicle-treated mice (Figure 3A, right panels). These results indicated that the more severe disease developed by H_4 receptor antagonist-treated mice was associated with more marked

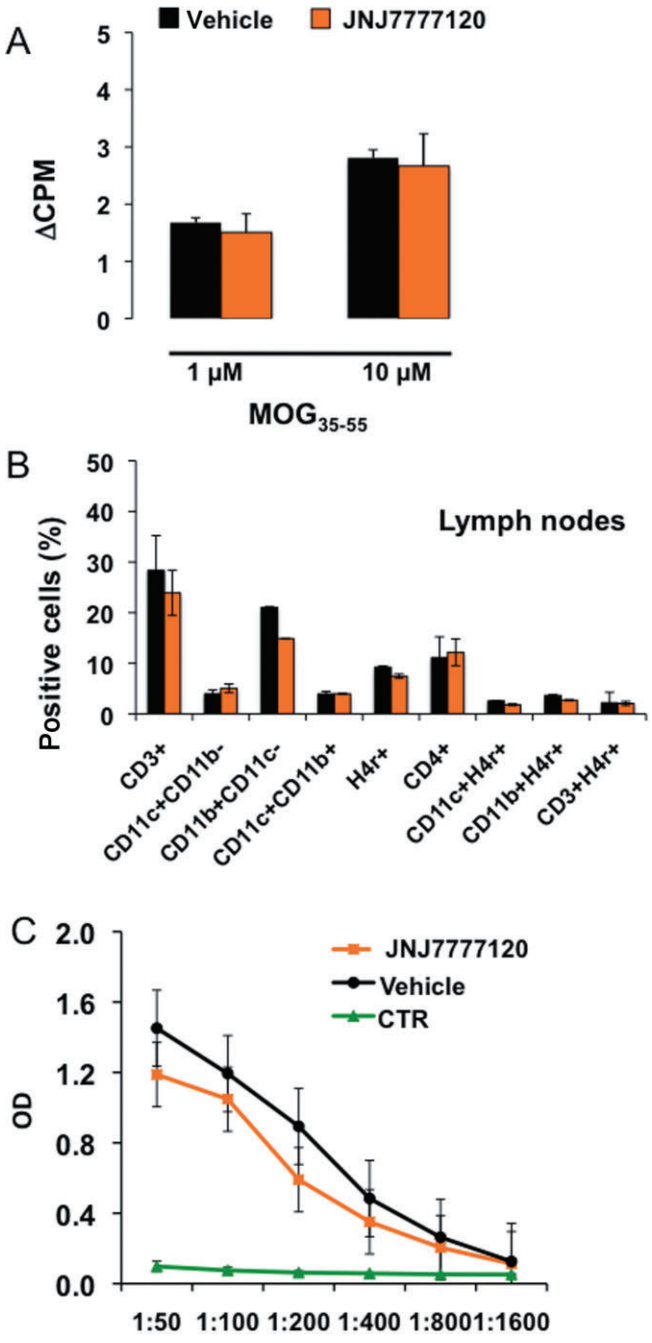

Figure 2

Effect of the H_4 receptor antagonist JNJ7777120 on post-EAE immune response. (A) *In vitro* proliferation of T lymphocytes isolated from LNs at D28 p.i., cells were incubated for 72 h with two doses of MOG_{35-55}. Proliferation was evaluated by thymidine incorporation measured during the last 12 h of culture. Data are expressed as ΔCPM (mean CPM stimulated cells – mean CPM background), $n = 3$ per group. (B) Flow cytometric analysis of cell distribution in LN at D28 p.i., cells freshly isolated from LN of three mice per group were labelled with monoclonal antibodies ($CD3^+$, T lymphocytes; $CD11b^+$, macrophages and NKs; $CD11c^+$, dendritic cells; $CD4^+$, T helper lymphocytes). All labelled cells were tested for surface expression of H_4R. (C) anti-MOG_{35-55} antibodies titrated by solid phase ELISA in individual sera of EAE-induced mice collected at D28 p.i., $n = 3$ per group. CTR, sera of non-immunized mice.

histological signs of injury in the spinal cord. Macrophage/microglia migration and activation in the CNS are critical for the demyelination and for clinical signs of EAE. As neuroinflammation and neurodegeneration are classical signs of EAE pathology, we then evaluated the presence of neuroinflammation in the spinal cord of EAE mice by immunodetection of GFAP-positive cells. In vehicle-treated mice, a small number of hypertrophic astrocytes were seen at D18 p.i. (Figure 3B, top panels). Few hypertrophic astrocytes were seen in vehicle-treated mice, whereas in JNJ7777120-treated mice, we observed an intense glial activation with cells intensely immunostained for GFAP, with enlarged cell bodies and long processes (Figure 3B, bottom panels).

To assess the presence of macrophage/microglia in the CNS of JNJ7777120- and vehicle-treated EAE mice, we immunostained spinal cord sections of EAE mice killed at D18 p.i., with Iba1 (a marker for macrophage/microglia activation) antibodies. A few Iba1-positive cells were observed within the inflammatory patches of vehicle-treated EAE mice (Figure 4A), whereas in JNJ7777120-treated EAE mice, several Iba1-positive cells with either thick, arborized processes or amoeboid shape, characteristic of activated microglia cells were found (Figure 4A). In addition, strong immunostaining for the proinflammatory cytokine IFN-γ was observed in the spinal cord of EAE mice treated with JNJ7777120 (Figure 4A). Hence, our data show an increased dissemination of activated microglia and production of proinflammatory cytokines within cell infiltrates in JNJ7777120-treated EAE mice. The analysis of $CD4^+$ T lymphocytes infiltrating the spinal cord showed a trend towards an increased percentage of $CD4^+H_4R^+$ in JNJ7777120-treated EAE mice, although it did not reach statistical significance (Figure 4B), suggesting that this lymphocyte subpopulation may be, in part, responsible for the increased severity of the disease.

Treatment with a H_4 receptor antagonist modifies cytokine production in EAE mice

To delineate the immune mechanism underlying increased EAE severity in JNJ7777120-treated EAE mice and confirm the immunohistochemical results, cytokine production was evaluated at D18 p.i. Cytokines such as IFN-γ have a prominent role in the inflammatory response mediated by CNS infiltrating lymphocytes during EAE (El-Behi *et al.*, 2007), whereas production of IL-10 and IL-4 moderates the inflammatory response. Hence, lymphocytes from MOG_{35-55}-immunized mice were re-stimulated with MOG_{35-55} *in vitro*. A significant difference in polarizing cytokine production was detected between JNJ7777120- and vehicle-treated lymphocytes (Figure 5A). Release of IFN-γ increased significantly in JNJ7777120-treated EAE mice compared with controls ($P = 0.01$, $n = 5$ 3, respectively), whereas significantly less IL-4 ($P = 0.042$) was produced. We observed a clear trend in IL-10 production, although the difference did not reach statistical significance ($P = 0.054$). No significant differences were found in IL-6 production ($P = 0.13$). All differences were seen after antigen-specific stimulation (MOG) and not after stimulation with a mitogen (PHA), underscoring the involvement of T-cells acting during autoimmune reaction.

Treatment with JNJ7777120 increased the percentage of IFN-γ^+ cells among gated $CD4^+$ lymphocytes ($n = 3$ vehicle, $n = 5$ JNJ7777120), but not among iNKT cells (Figure 5B), early

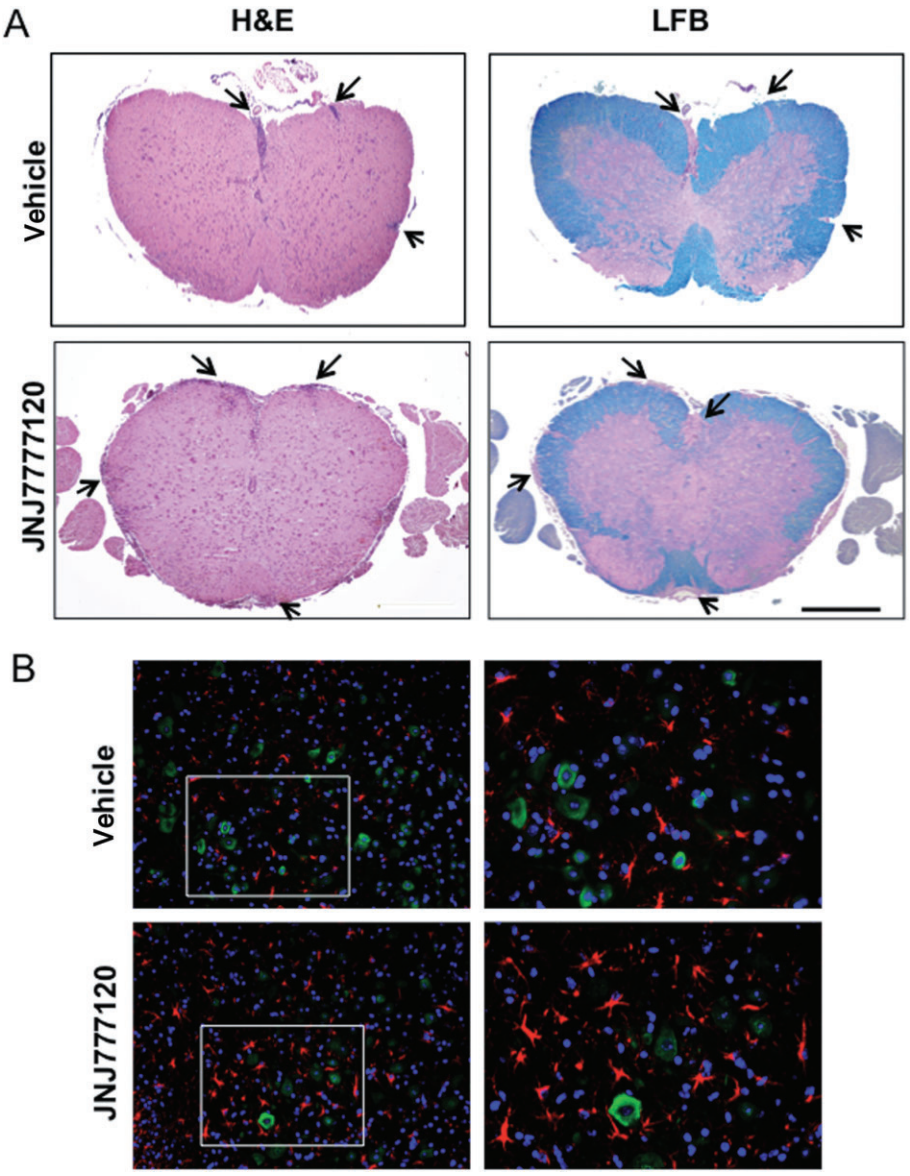

Figure 3

Effect of the H_4 receptor antagonist JNJ7777120 on the histopathology and on astroglia of EAE mice. (A) Consecutive sections were stained for H&E or LFB and examined at 10× magnification to detect inflammatory infiltrates and demyelination, respectively, as indicated by arrows. Representative photomicrographs of spinal cord sections show larger and more numerous areas of inflammatory infiltrates in JNJ7777120-treated than in vehicle-treated EAE mice as indicated by arrows. Note the presence of larger plaques of demyelination (right panels) in the same areas of infiltrates shown in the left panels. Scale bar: 500 μm. (B) Sections were stained with anti-GFAP antibody (red) and with NeuN (green) to detect astrocytes and neurons, respectively, and counterstained with DAPI (blue). Right panels show magnifications of the white frames in left panels. GFAP immunoreactivity was more intense in JNJ7777120-treated EAE mice as astrocytes were more numerous with enlarged cell bodies and long processes. All experiments were performed on EAE mice killed at D28 p.i. with comparable scores (<2.0).

players in acute neuroinflammation (Singh *et al.*, 2001; Denney *et al.*, 2012) that are modulated by activation of H_4 receptors (Leite-de-Moraes *et al.*, 2009).

Treatment with a H_4 receptor antagonist modifies transcription factor expression in EAE mice

In order to evaluate if the modulation of cytokines production was due to the activation of different subsets of T $CD4^+$ lymphocytes, we analysed the relative expression of Tbet, GATA3, FOXP3 and RORc, transcription factors that affect the functional capabilities and flexibility of $CD4^+$ T-cell subsets (Oestreich and Weinmann, 2012), in T-cells isolated from LNs of EAE mice at D18 p.i. Tbet and FOXP3 were significantly increased ($P = 0.02$ and $P = 0.03$, respectively) in JNJ7777120-treated EAE mice, whereas increase of GATA3 and RORc did not reach statistical significance (Figure 6A). When expression of transcription factors was analysed as the ratio (see

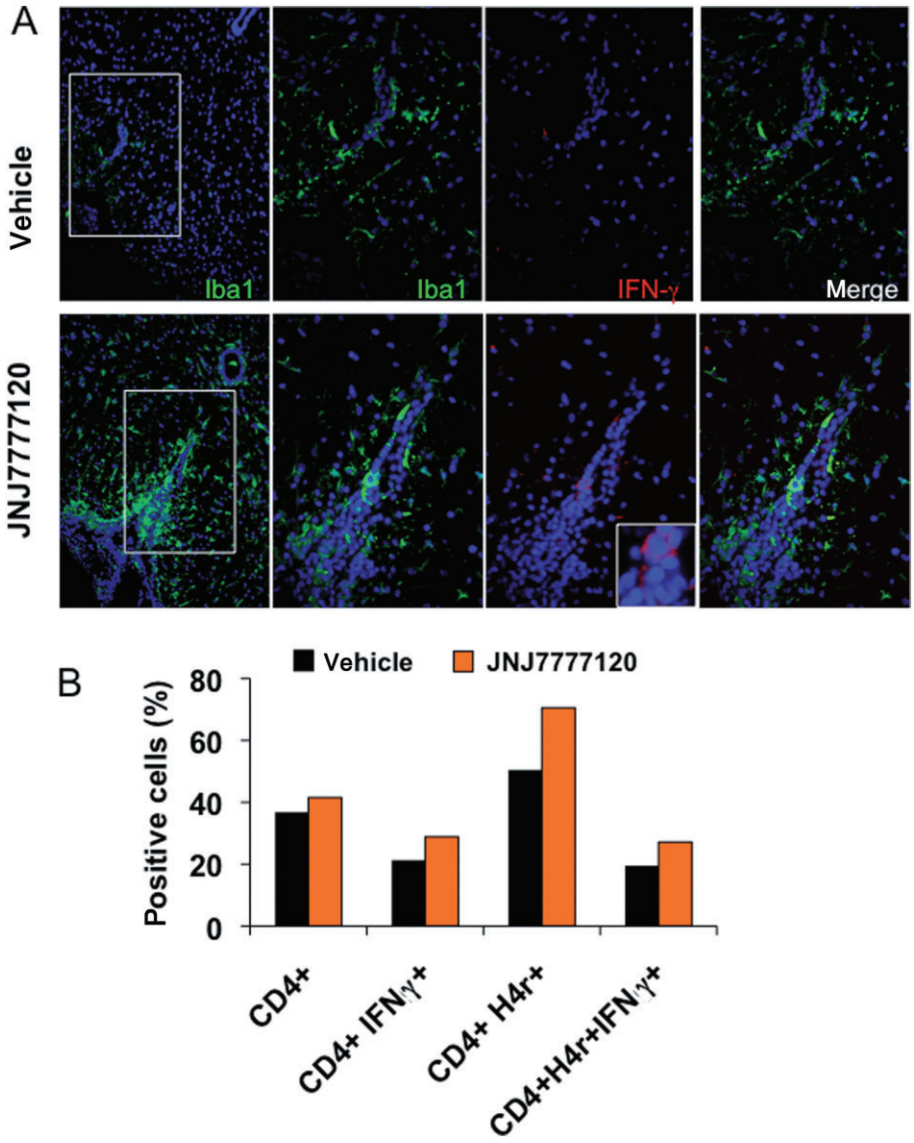

Figure 4

Effect of the H_4 receptor antagonist JNJ7777120 on microglial activation in the spinal cord of EAE mice. Shown are representative photomicrographs of coronal spinal cord sections from vehicle- and JNJ7777120-treated EAE mice. (A) Sections were double-labelled with anti-Iba1 antibodies (green) to detect microglia and macrophages and anti-IFN-γ antibodies (red) and counterstained with DAPI (blue). Sections were examined at 20× and 40× magnification. All experiments were performed on mice (D28 p.i.) with comparable scores (<2.0). (B) Flow cytometric evaluation of freshly isolated spinal cord infiltrates at D18 p.i. Data are percent of CD4/IFN-γ/ H_4 receptor-positive cells in a pool of three vehicle- and three JNJ7777120-treated mice.

Methods) between two single factors, we noticed a difference in the FOXP3/RORc values, suggesting a simultaneous increase of regulatory T-cells and a decrease in Th17 subtypes in vehicle-treated mice (Figure 6B). These data are in agreement with decreased IL-17A mRNA production in vehicle-treated mice (Figure 6C).

Discussion

In this study, we report that blockade of the histamine H_4 receptor during the effector phase induced a more severe $MOG_{35–55}$-induced EAE because mice treated with a selective H_4 receptor antagonist JNJ7777120 exhibited an exacerbation of disease and more severe histopathological features, as well as dysregulation of cytokine production. Histamine participates in the development and progression of autoimmune inflammatory diseases such as MS and EAE (Jadidi-Niaragh and Mirshafiey, 2010), although, to date, there is only circumstantial evidence that H_1 receptors modulate susceptibility to MS, in a small cohort of patients (Logothetis *et al.*, 2005). As shown in preclinical studies, all four histamine receptors participate in disease development and EAE susceptibility either by regulating function of APCs, encephalito-

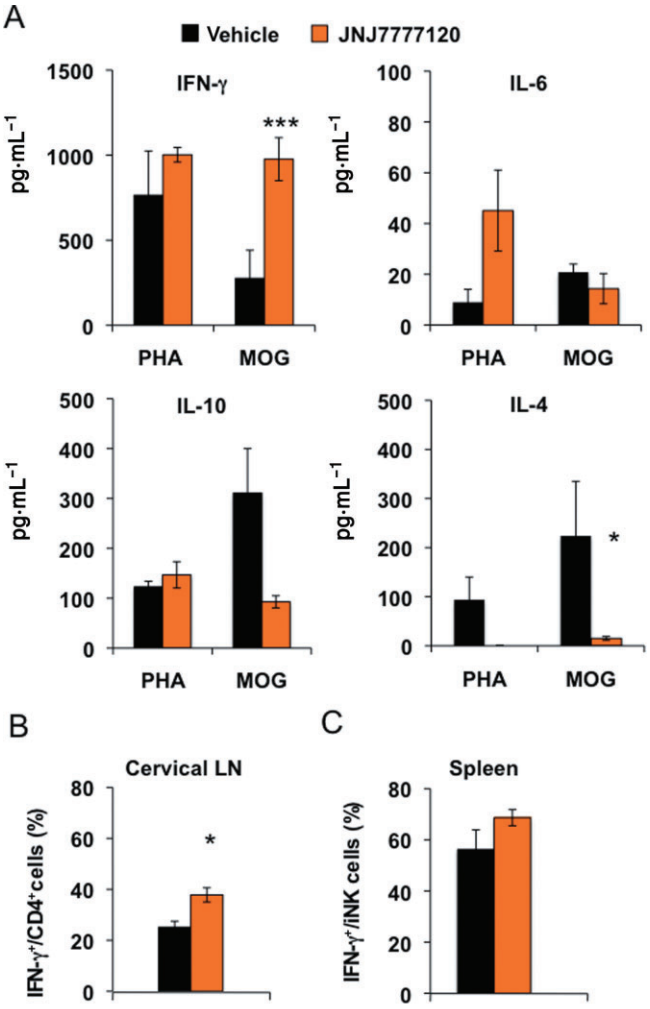

Figure 5

Effect of the H_4 receptor antagonist JNJ7777120 on cytokine production in EAE mice. (A) ELISA determination of IFN-γ, IL-6, IL-10 and IL-4 in supernatants of LN cells isolated at D18 p.i. and stimulated with PHA (5 μg·mL^{-1}) or $MOG_{35–55}$ (50 μg·mL^{-1}). n = 3 for vehicle and n = 5 for JNJ7777120-treated mice. *P < 0.05; ***P < 0.001; unpaired Student's t-test. (B) Flow cytometric analysis of CD4/IFN-γ-positive cervical LN cells isolated at D18 p.i. and of (C) spleen iNK cells. n = 3 for vehicle and n = 5 for JNJ7777120-treated mice. Data are expressed as % of positive cells.

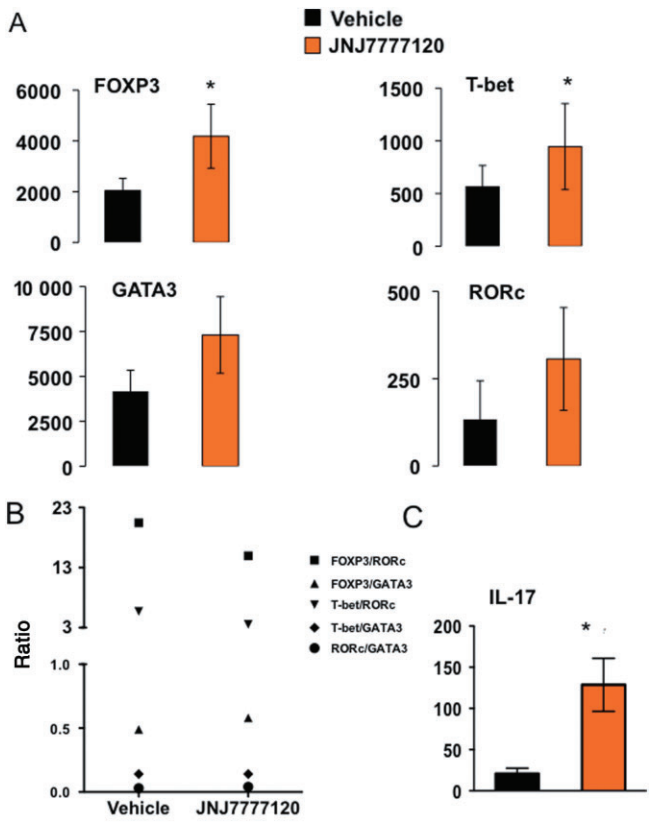

Figure 6

Effect of the H_4 receptor antagonist JNJ7777120 on Th cell transcription factors and IL-17 expression. mRNAs extracted from draining lymphocytes from vehicle- and JNJ7777120-treated mice at D18 p.i. were reverse transcribed and the gene expression level determined by qRT-PCR using gene-specific primers for indicated genes and ubiquitin carboxy-terminal hydrolase L1 (HPRT-1, hypoxanthine phosphoribosyltransferase 1) as a housekeeping gene for normalization. (A) Relative expression of mRNA levels of FOXP3, T-bet, RORc and GATA3. (B) Each point represents the mean of designated transcription factors calculated within each animal and then averaged within experimental group. (C) Relative expression of mRNA levels of IL-17. n = 3 vehicle-treated mice; n = 4 JNJ7777120-treated mice. *P < 0.05; unpaired t-test.

genic T-cell responses or blood–brain barrier permeability. However, these studies revealed intriguing and somewhat unexpected effects of histamine receptor activation or blockade, depending on the components of the immune response that were analysed, whether in genetically modified mice, *ex vivo* or *in vivo* models (see Passani and Blandina, 2011). The distribution of the H_4 receptor on haematopoietic cells and its primary role in inflammatory functions have made it a very attractive target for the treatment of asthma and refractory inflammation (Bhatt *et al.*, 2010). Hence, there is great interest in therapies based on new selective ligands of the H_4 receptor. The H_4 receptor antagonist JNJ7777120 has been extensively used to elucidate the roles of H_4 receptors in a variety of allergic and inflammatory processes, mainly in *ex vivo* or *in vitro* assays, less so for *in vivo* treatments. In heterologous systems transfected with human H_4 receptors, JNJ7777120 displayed biased signalling (Seifert *et al.*, 2011) by selectively recruiting β-arrestin2 (Rosethorne and Charlton, 2011), a feature shared by several H_4 receptor ligands (Nijmeijer *et al.*, 2012). In an integrated system, JNJ7777120 antagonizes variable levels of endogenous histamine; therefore, it is hard to predict if ligand-specific signalling bias may have important therapeutic implications.

Here, we show that during disease progression, T lymphocytes of JNJ7777120-treated EAE mice produced more IFN-γ than vehicle-treated controls, although their frequency and proliferative capacity in response to $MOG_{35–55}$ remained unchanged. These cells also showed a clear trend towards lower levels of regulatory cytokines such as IL-4 and IL-10, although this last effect did not reach statistical significance.

Our results are in agreement with the recent report that mice which do not express H_4 receptors exhibit an exacerbated disease and immunopathology (del Rio *et al.*, 2012), although the sequence of events during induction and progression of the disease may differ. In our experiments, antagonism of H_4 receptors at the onset of the disease seems to affect more strongly the effector arm of the immune response, that is, IFN-γ-producing CD^+ T-cells (i.e. Th1/Th17 cells), than the IL-10 producing regulatory side. In contrast, lack of H_4 receptor signalling in H_4R-KO mice affects the frequency and suppressive activity of Treg cells during the early acute phase of EAE, leading to impairment of anti-inflammatory response and increased proportion of Th17 in the CNS, but not of Th1 (del Rio *et al.*, 2012).

The analysis of transcription factors showed a significant increase of FOXP3 and Tbet expression. Although it is commonly accepted that FOXP3 and Tbet expression identifies Treg and Th1 cells, respectively, expression of a single transcription factor is not sufficient to characterize the Th phenotype of $CD4^+$ T-cells (Oestreich and Weinmann, 2012). Indeed, increased FOXP3 expression may indicate a generalized increase of T lymphocyte activation state. Compelling data suggest that FOXP3 is expressed by recently activated T-cells and it has been hypothesized that a cell population with positive FOXP3 together with other line-specific transcription factors represents a plastic cell population that, during the EAE autoimmune reaction, migrates from the LNs to the CNS (Pillai *et al.*, 2007; Esposito *et al.*, 2010). Therefore, in the context of increased production of IFN-γ, our observations indicate that the enhanced activation of Th1 or Th1/Th17 cells was responsible for the more severe disease. At the same time, our results showed a decreased ratio of FOXP3/RORc (Treg/Th17 axis), suggesting a trend towards Th17 polarization, confirmed by a significant increase of IL-17 mRNA expression in draining LNs of JNJ7777120-treated EAE mice. All together, these data indicate that H_4 receptor signalling has a role in determining the proportion of Th1/Th17 during EAE, and it will be crucial to clarify the extent to which this phenomenon is secondary to the modulation of regulatory mechanism in EAE.

It has been reported that antagonism of H_4 receptors determines iNKT cell deficits, similar to that observed in histidine decarboxylase- and H_4R-KO mice (Leite-de-Moraes *et al.*, 2009). iNKT cells, a small group of circulating mature T lymphocytes, control immune responses by the production of several cytokines, such as IL-4 and IFN-γ, that depends on H_4 receptor signalling. During the acute phase of EAE, activation of iNKT cells may influence the occurrence of infiltrating inflammatory monocytes and activation of Th1/Th17 cell, therefore modulating disease severity (Singh *et al.*, 2001; Furlan *et al.*, 2003). We found that treatment of EAE mice with JNJ7777120 did not change the proportion of activated iNKT cells, suggesting that they did not contribute significantly to the increased severity of disease.

Recent evidence has shown the topological and functional localization of H_4 receptors in the CNS (Strakhova *et al.*, 2009), which have been detected on the soma of small and medium diameter sensory neurons, as well as in lamina I-II of the lumbar spinal cord (Connelly *et al.*, 2009) and on microglia (Ferreira *et al.*, 2012). Therefore, the possibility that blockade of central H_4 receptors contributes to the exacerbation of EAE cannot be excluded.

It is becoming clear that the role of H_4 receptors in immune disease is context-specific, depending on the animal model used and the complement of H_4 receptor-expressing cells recruited at the onset of autoimmune diseases. As recently pointed out by Mommert *et al.*, (2011), activation of H_4 receptors on Th cells may enhance release of proinflammatory cytokines and promote inflammation, whereas it may control inflammation by suppressing the production of cytokines and chemokines in APCs, thus influencing Th-cell polarization linking innate and adaptive immune pathways. In the context of the conflicting activities of H_4 receptors, additional research is required to better foresee the possible clinical benefits and detrimental effects in the treatment of inflammatory or autoimmune diseases with H_4 receptor ligands.

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Conflict of interest

The authors declare no conflicts of interest.

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