# Identification of the Role of the New Histamine 4 Receptor in Inflammation and Immunity

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Jeffery Mark COWDEN

# Contents

# Abbreviations

AD, Atopic Dermatitis

Dex, dexamethasone

CAIA, Collagen Antibody Induced Arthritis

CIA, Collagen Induced Arthritis

Fex, fexofenadine.

FITC, Fluorescein isothiocyanate

$H_XR$, histamine X receptor where X indicates the receptor number

KO, Knock Out

p.o., orally (per os)

RA, Rheumatoid Arthritis

WT, Wild Type

# Abstract

**General Introduction:** Histamine is a biologically active amine which involved in local immune responses as well as regulating diverse physiological functions. Histamine exerts its function through corresponding receptors. Physiological and pharmacological profiling predicted that histamine receptor consists of several subtypes. Two of major histamine receptor subtypes, $H_1R$ and $H_2R$, have already been well studied pharmacologically; and $H_1R$ antagonists have been developed as drugs to treat allergic rhinitis, atopic dermatitis (AD), insomnia. On the other hand, $H_2R$ antagonists are used as drug to treat heart burn and acid reflux. It was highly expected that there might be additional histamine receptor subtypes through pharmacological studies; and $H_3R$ and $H_4R$ were identified by molecular biological approaches. The function of $H_3R$ has already expected to involve the central and peripheral regulation of the levels of histamine and other neurotransmitters, however, there was nearly no known information about $H_4R$ function. $H_4R$ is expressed mainly on cells of the immune system so I studied the role of $H_4R$ focused on its involvement in the inflammation and immunity.

**Part 1:** Atopic dermatitis (AD) is a disease were traditional $H_1R$ antagonists are primarily used, but the efficacy of $H_1R$ antagonist is not sufficient and there is need for better therapies. My studies focused on the role of H4R in dendritic cell migration to the lymph node and a reduction of T cell responses to antigen stimulation resulting in decreased inflammation in the skin. Ongoing pruritus is a common comorbidity of AD, and I have identified superior efficacy of $H_4R$ antagonist to $H_1R$ antagonist in controlling pruritus in preclinical disease model.

**Part 2:** I have studied on the role of $H_4R$ in autoimmunity, especially in Rheumatoid arthritis (RA). Although elevated histamine levels occur in RA patients, they do not respond to traditional $H_1R$ or $H_2R$ antagonists. I have identified that $H_4R$ with its expression in immune cells could pay a pivotal role in preclinical disease models. $H_4R$ was involved in both innate and adaptive immunity in preclinical RA models and contributed to the recruitment of mast cell to the inflamed synovium and reduction of key cytokines such as IL-17.

**General Discussion:** My studies have identified novel role of H4R in physiological and pathophysiological conditions. The AD study (Part 1) demonstrated $H_4R$ antagonists are anti-pruritic and anti-inflammatory in a mouse

model of Th2-dependent skin inflammation. This effect was superior to that of existing $H_1R$ antagonists. The anti-inflammatory properties appeared to be driven by a reduction in Th2 cell activation that can be partially accounted for by a reduction in the migration of antigen-bearing dendritic cells to the lymph nodes. Therefore, the effects of the $H_4R$ antagonists on pruritus, inflammation, and Th2-cell responses point to their therapeutic potential for the treatment of inflammatory skin disorders such as AD. The other studies focused on RA (Part 2) clearly showed a role of $H_4R$ in arthritis. Importantly, the receptor has effects in both a model of inflammatory arthritis and one of autoimmune arthritis, suggesting that $H_4R$ can affect both innate and adaptive immune responses. Taken as a whole, my study suggests that the $H_4R$ can be not only the initiator of inflammation, but also potentiator of inflammatory responses. Therefore, antagonism of $H_4R$ would not be expected to be immunosuppressive, but rather lead to a dampening of the initial inflammatory response, thereby leading to a reduction in inflammation in a variety of disease states, including allergic or autoimmune. I would also discuss on the recent data from a human phase II trial demonstrating $H_4R$ antagonists reduce histamine-induced pruritus in AD patients. As a whole, my research on $H_4R$ has significantly contributed to increase our understanding on histamine and $H_4R$ and how they modify immunity and inflammation.

# General Introduction

Histamine is a biologically active amine which involved in local immune responses as well as regulating diverse physiological functions. Histamine exerts its function through corresponding G protein coupled receptors. Physiological and pharmacological profiling predicted there would be more than one single histamine receptor. Two of major histamine receptor subtypes, $H_1R$ and $H_2R$, were identified in the 1940s and thus had already been well studied. Antagonists drugs $H_1R$ to have been developed as drugs to treat allergic rhinitis, atopic dermatitis (AD), and insomnia. On the other hand, $H_2R$ antagonists are used as drug to treat heart burn and acid reflux. In fact Nobel prizes were award to Daniel Bovet in 1957 for the discovery of $H_1R$ antagonists and to Sir James Black in 1988 for the identification $H_2R$ antagonists. However, it was highly expected that there might be additional histamine receptor subtypes as histamine still produced pharmacology when both $H_1R$ and $H_2R$ were blocked with antagonist. The $H_3R$ in 1999 and $H_4R$ in 2000 were identified by molecular biological approaches. The function of $H_3R$ is still being characterized but as it express on in the central nervous system it likely plays a role in regulating neurotransmitters. $H_4R$ is expressed in both the central nervous system and more interestingly on immune cells. In the mid-2000s knock out mice and antagonists were generated as tool to allow the characterization of the fourth histamine receptor. There was nearly no known information about $H_4R$ function. Because of $H_4R$ is expression mainly on immune cells the first works focused is role in inflammation and immunity. Within this works focus is on the discovery the role $H_4R$ plays in the pathogenesis of atopic dermatitis (AD) and Rheumatoid Arthritis (RA). However, it should be noted others and myself have also characterized $H_4R$ role in pain, asthma, and colitis.

AD is an extremely common disease effecting 20% of people at some part of their life. Although generally a disease of childhood that patients outgrow, it does effect some patients throughout their lives. The disease is characterized by itchy, red, swollen and cracked skin. In children it can be present anywhere, but in adults it's general found on the hands, feet, knees and elbows. The disease can be exacerbated by frequent bathing, and the desire to itch areas of the effected skin. AD a disease where traditional $H_1R$ antagonists are primarily used, but the efficacy of $H_1R$ antagonist is not sufficient and there is need for better therapies. Here within are the results of studies identifying for the first time the role $H_4R$ in AD. Specifically how $H_4R$ antagonists affects dendritic cell migration to the lymph node results in a reduction of T cell responses to antigen stimulation. This leads to decreased inflammation in the skin pre-clinically. Ongoing pruritus is a common comorbidity of AD and not controlled by

current therapies. Yet, in these studies $H_4R$ antagonists have superior efficacy to a $H_1R$ antagonist in controlling pruritus in a preclinical disease model.

Rheumatoid arthritis is a chronic progressive autoimmune disease that attacks the joints of patients. Although the cause is unknown genetic and environmental factors play a role leading to 0.5 to 1% of the population being effected. The ongoing inflammation in the joints can be severe leading to destruction of the joint and loss of mobility or functional use of the hands. Promising drugs such as anti-TNF-a antibodies have been developed over the years to slow the course of the disease they are not curative and for unknown reason they become ineffective in patients after several years of use. New curative treatments or treatment for patients where current therapies are ineffective are needed. Although elevated histamine levels occur in RA patients, they do not respond to traditional $H_1R$ or $H_2R$ antagonists. Yet again $H_4R$ with its expression in immune cells could pay in autoimmune disease like RA. Here within find the results of studies were $H_4R$ was involved in both innate and adaptive immunity in preclinical RA models and contributed to the recruitment of mast cell to the inflamed synovium and reduction of key cytokines such as IL-17. Treatment with $H_4R$ antagonist resulted in greatly reduced RA like disease severity in two pre-clinical models of RA. In addition, the TH17 cytokine was greatly reduce likely be one of several mechanism of the efficacy.

# Part 1: $H_4R$ and Atopic Dermatitis

# Abstract

The role of histamine $H_4$ receptor ($H_4R$) was investigated in a Th2 cell-mediated mouse skin inflammation model that mimics several of the features of atopic dermatitis. Treatment with two specific $H_4R$ antagonists before challenge with fluorescein isothiocyanate (FITC) led to a significant reduction in ear edema, inflammation mast cell and eosinophil infiltration. This was accompanied by a reduction in the levels of several cytokines and chemokines in the ear tissue. Upon *ex vivo* antigen stimulation of lymph nodes, $H_4R$ antagonism reduced lymphocyte proliferation and the levels of IL-4, IL-5 and IL-17. One explanation for this finding is that lymph nodes from animals dosed with the $H_4R$ antagonist, JNJ 7777120, contained a lower number of FITC positive dendritic cells. The effect of $H_4R$ antagonism on dendritic cell migration *in vivo* may be an indirect result of the reduction in tissue cytokines and chemokines or a direct effect on chemotaxis. In addition to anti-inflammatory effects, JNJ 7777120 also significantly inhibited the pruritus exhibited in the model. Therefore, the dual effects of $H_4R$ antagonists on pruritus and Th2 cell mediated inflammation point to their therapeutic potential for the treatment of Th2-mediated skin disorders including atopic dermatitis.

# Introduction

The histamine $H_4$ receptor ($H_4R$) is the most recently described histamine receptor and is primarily expressed on hematopoietic cells, in particular, dendritic cells, mast cells, and eosinophils(for a recent review see Huang and Thurmond 2008). Notably, the $H_4R$ exerts profound effects in regulating immune cell functions, i.e. chemotaxis, cytokine and chemokine expression (Huang and Thurmond 2008). A number of *in vivo* studies have demonstrated $H_4R$ involvement in both innate and adaptive immune responses (Coruzzi et al. 2007; Dunford et al. 2006; Thurmond et al. 2004; Varga et al. 2005). In particular the $H_4R$ has been shown to mediate Th2 responses *in vivo* and in vitro and antagonists of the receptor reduce lung inflammation in a mouse model of allergic asthma (Dunford et al. 2006). These, and other lines of evidence, make $H_4R$ a promising immunomodulatory target for the treatment of allergic, autoimmune, and other inflammatory diseases (Huang and Thurmond 2008; Thurmond, Gelfand, and Dunford 2008). In addition to its effects in inflammation, the $H_4R$ has also been shown to direct pruritic responses in mice (Bell, McQueen, and Rees 2004; Dunford et al. 2007; Rossbach et al. 2009). This dual effect on allergic inflammation and pruritus suggests that the $H_4R$ is a promising new therapeutic target for treating allergic skin diseases such as atopic dermatitis.

Atopic dermatitis is a common chronic inflammatory skin disease with symptoms that include skin lesions, pruritus and dry skin (for recent reviews see Akdis et al. 2006; Bieber 2008; Homey et al. 2006). The condition is often predictive of subsequent atopic disorders such as allergic rhinitis and asthma. All atopic diseases share certain pathogenic and immunologic elements such as eosinophilia and elevated IgE levels (Spergel and Paller 2003; Cookson 2004; Avgerinou et al. 2008). Atopic dermatitis is thought to be driven, at least in the early stages, by Th2 cell responses, since lesions exhibit marked T cell infiltration and these cells predominantly express

IL-4, IL-5 and IL-13, especially during the acute phase, but Th1 responses may play a more dominant role in chronic lesions (Akdis et al. 2006; Bieber 2008; Homey et al. 2006).

Histamine is recognized as a major inflammatory mediator released by mast cells, basophils, and other cells during allergic reactions and exerts its actions through four distinct G-protein-coupled receptors. An increase in histamine levels has been noted in the skin and plasma of atopic dermatitis patients (Johnson et al. 1960; Juhlin 1967) and basophils and mast cells are increased in atopic dermatitis lesions (Jarvikallio et al. 1997; Phanuphak et al. 1980; Horsmanheimo et al. 1994). Antihistamines that target the histamine $H_1$ receptor ($H_1R$) are frequently used for the relief of the associated pruritus, but their effectiveness appears to be restricted to the first generation sedating $H_1R$ antagonists and non-sedating antihistamines have little benefit (Akdis et al. 2006). This suggests that either histamine is not involved in the disease pathophysiology or that receptors other than the $H_1R$ may be important in histamine-mediated responses in atopic dermatitis.

To explore this I have used two potent and specific $H_4R$ antagonists to examine the role of $H_4R$ in mediating inflammation and pruritus in a Th2 cell-mediated mouse skin inflammation model. The FITC skin model used here is a contact dermatitis model, but it has been shown to be IgE, Th2 cytokine and CD4+ T cell dependent and is characterized by strong eosinophilia, unlike other Th2 sensitizers (Dearman and Kimber 2000; Takeshita et al. 2004). Thus, this model has several features similar to atopic dermatitis in humans.

# Materials and Methods

**Mice**

BALB/c mice were obtained from Charles River Laboratories (Wilmington, MA). WBB6F1 +/+ and WBBF1 $W/W^v$ mice were from Jackson Laboratory (Bar Harbor, Maine). $H_4R$ deficient mice were generated as previously described (Hofstra et al. 2003) and crossed on to a BALB/c background for at least ten generations. Age matched animals were used in all experiments. Mice were housed in community cages on a 12 h light cycle and fed mouse chow and water ad libitum. All procedures were performed according to the internationally accepted guidelines for the care and use of laboratory animals in research and were approved by the local IACUC.

**Materials**

JNJ 7777120, (5-Chloro-1H-indol-2-yl)-(4-methyl-piperazin-1-yl)-methanone), and JNJ 28307474, 5-Fluoro-4-methyl-2--1H-benzoimidazole, were synthesized as previously described (Arienti et al. 2005; Jablonowski et al. 2003). Fluorescein isothiocyanate (FITC), dexamethasone, fexofenadine and dibutylphthalate were obtained from Sigma-Aldrich, Inc. (St. Louis, MO). The selectivity of JNJ 7777120 has been previously described (Thurmond et al. 2004). The selectivity of JNJ 28307474 is given in Tables 1- 3. The binding assays were carried out as previously described (Thurmond et al. 2004).

**FITC model**

Female mice (6 to 8 wk old) were used. The abdomen of each animal was shaved and sensitized by the application of 100 μl of FITC in dibutylphthalate and acetone on two consecutive days. Five days after the sensitization, the baseline thickness of the ears was measured using calipers

with the animals under light isofluorane anesthesia. One ear was then painted with 15 μl of FITC and the contra-lateral ear was painted with vehicle (dibutylphthalate /acetone). Finally, the thickness of the ears was measured again 24 h after FITC application. Animals were then euthanized and biopsies of the ear collected. In addition, a repeat sensitization model was also performed in which animals were sensitized on days 1, 2 and 15, 16 and challenged on day 21. The thickness of the ears was measured again 24 h after FITC application.

**Compound administration**

All compounds were formulated in 2-hydroxypropyl-β-cyclodextrin for all experiments and were administered per os by oral gavage (p.o.) 20 min prior to FITC challenge and 4 hours post challenge.

**Histology**

For histological examination, ear specimens were fixed in 10% buffered formalin and embedded longitudinally in paraffin by standard methods. Four-micron sections were stained with Wright-Giemsa stain. Individual parameters such as inflammation, edema and the number of abscesses were assessed and scored as follows:

Inflammation

0- No visible inflammation

1- Inflammatory cells present along less than 40% of the length of the skin

2- Inflammatory cells present between 40 and 80% of the length of the skin

3- Inflammatory cells present along greater than 80% of the length of the skin

Edema

0- No edema

1- Increase in relative thickness from normal by 20%

2- Increase in relative thickness from normal by 20 to 40%

3- Increase in relative thickness from normal more than 40%

Abscesses

0- No abscesses

1- Less than 2

2- Less than 4

3- Greater than or equal to 4

A total severity score was obtained by adding the scores from the three assessments above. The theoretical maximum severity score was nine. The score was determined from 6 whole sections from each animal. In addition the number of eosinophils and mast cells were quantitated from 8 randomly selected high-power fields from 4 sections for each animal.

**Cytokine measurements in ear tissue**

Skin ear biopsies were pooled from four test animals. Biopsies were minced and then repeatedly homogenized with beads in PBS plus Complete$^{TM}$ protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN) at 4°C. Supernatant was collected and analyzed for the presence of cytokines using a Luminex multiplex system (Luminex Corp., Austin TX) with a Mouse Cytokine LINCOplex Panel (Millipore, Billerica, MA) as per manufacturer's protocol (mouse twenty-two cytokine kit).

**FITC-specific T cell responses *in vitro***

Auricular lymph node cells were isolated from immunized mice, pooled and cultured in quadruplicate (5 X $10^5$ cells/well) with medium (RPMI 1640 supplemented with 10% fetal bovine serum, non-essential amino acids and β-mercaptoethanol) alone or with medium plus 10 µg/mL FITC (diluted from 10 mg/mL stock in 100% DMSO) for 96 h. Cell culture supernatants were collected after three days, and cells were continued in culture with [$^3$H]-Thymidine (1 µCi/well) for another 18 h for proliferation assays. [$^3$H]-Thymidine uptake was quantitated by liquid scintillation counting. Cytokine levels in cell culture supernatants were determined using a Luminex multiplex system (Luminex Corp., Austin TX) with a Bio-Rad Bioplex (Hercules, CA) or Mouse Cytokine LINCOplex Panel (Millipore, Billerica, MA) as per manufacturer's protocol

(mouse eighteen or twenty-two cytokine kit, respectively). *For anti-CD3/anti-CD28 stimulations,* 96-well plates were coated with 100 µl/well of 10 µg/ml anti-mouse CD3 (NA/LE) (BD Pharmingen, San Diego, CA) in PBS, incubated at 4$^{o}$C overnight. Before adding cells the wells were aspirated and washed twice with PBS. After the addition of cells (5 x $10^{5}$ cells/well), anti-mouse CD28 (NA/LE) (BD Pharmingen, San Diego, CA) was added to a final concentration of 2 µg/ml and incubated for 72 h before processing as above for proliferation and cytokine production.

**Dendritic cell migration *in vivo***

Mice were shaved and sensitized by application of 100 µl of 0.5% FITC in dibutylphthalate and acetone onto the abdomen on two consecutive days. Five days after the sensitization, animals were dosed with compounds, and 30 min later, one ear was painted with 15 µl of 0.5% FITC on both sides and the control ear was painted with vehicle (dibutylphthalate /acetone). Twenty-four hours after the application of FITC, mouse auricular lymph nodes were removed, and digested with 2.5 ml digestion buffer (RPMI 1640 containing 50 µl of DNase I (Sigma-Aldrich, Inc., St. Louis, MO) and liberase III (Roche Applied Science, Indianapolis, IN)) for 20 min at 37$^{o}$C. Fresh digestion buffer, 2.5 ml, was added and incubated for another 15 min at 37$^{o}$C. Lymph nodes were gently dispersed with a 70 µm cell strainer, flushed with 5 ml PBS containing 5 mM EDTA, spun down, washed once with 5 ml PBS containing 5 mM EDTA, and resuspended in 10 ml FACS buffer (PBS with 1% fetal calf serum). Cells were counted and adjusted to 1 x $10^{7}$ cells/ml and 1-2 x $10^{6}$ cells were used for each staining. Cells were incubated with 1:50 FcR blocker (BD Pharmingen, San Diego, CA) on ice for 15 min, stained with 1:50 PE-anti-CD11c or PE-anti-I-A$^{d}$ (BD Pharmingen, San Diego, CA) for 45 min on ice. Cells were washed once with

3 ml FACS buffer, resuspended, and collected on FACSCalibur (BD Biosciences Immunocytometry Systems, San Jose, CA). Right before analysis, 1 µg/ml propidium iodide was added to stain for dead cells.

**Pruritus Assessment**

Pruritus was quantified via counting of the number of bouts of scratching in a 15-min period starting 10 min after the application of FITC to the ear. Bouts of scratching were recorded and defined as previously described (Dunford et al. 2007).

# Results

**Histamine H4 receptor antagonism inhibits edema in a dermal inflammation model.**

Dermal inflammation was induced in Balb/C mice by topical exposure to fluorescein isothiocyanate (FITC) (Figure 1a). Mice were sensitized to FITC by painting on the abdomen on two consecutive days. Five days later FITC was applied to one ear and 24 h later the ear edema was evaluated as an indication of inflammation. The $H_4R$-selective antagonist (Thurmond et al. 2004), JNJ 7777120, administered 20 min prior to and 4 h after FITC application reduced the ear edema in a dose dependent fashion (Figure 1b). The maximum inhibition seen was 39% and higher doses did not yield any further reduction. This level of inhibition was similar to that seen using dexamethasone dosed at 3 mg/kg p.o. The role of the $H_4R$ in this model is further supported by the reduction in ear edema in mice deficient in the $H_4R$ compared to wild-type mice (Figure 1c). In further support of an $H_4R$ specific affect a second $H_4R$ antagonist, JNJ 28307474 (Tables 1 -3), of a completely different chemical class was also studied. This compound also inhibited ear edema formation with a maximal effect similar to that found with JNJ 7777120 (Figure 1d). While this compound does have some cross-reactivity with muscarinic and 5-HT receptors (Tables 2 and 3), the only affinity it shares in common with JNJ 7777120 is at the $H_4R$ (Thurmond et al. 2004) supporting the conclusion that the effects seen are mediated via the $H_4R$.

The inflammation in this model was also assessed by histopathological analysis of the ears. Twenty-four hours after FITC challenge there was significant inflammation. This consisted of mainly neutrophils, but there was also an increase in the number of mast cells and eosinophils in the skin (Figures 2- 3). The increase in mast cells and eosinophils is consistent with a Th2 response in this model. Treatment with the $H_4R$ antagonist led to a reduction both in the total severity score and in the number of eosinophils and mast cells (Figures 2- 3).

**Analysis of cytokines and chemokines**

A full time course was carried out and all of the cytokine and chemokines levels peaked at 12-18 h (Figure 4). Application of FITC lead to increases in IL-4 while IFN-ɣ levels were low (30-50 pg/ml) and did not change with FITC treatment (data not shown). This, along with the observed eosinophilia, supports the previous findings that this model elicits a Th2 response (Dearman and Kimber 2000; Takeshita et al. 2004). In addition there were increases in GM-CSF, IL-1β, IL-6, TN-Fα, RANTES, MCP-1, MIP-1α and KC. IL-2, IL-9 and IL-12 were detected at low levels and there was no change between control and FITC treated ears (data not shown). IL-10, IL-5 and IL-13 in the ear homogenates were too low to measure. Treatment with the $H_4R$ antagonist, JNJ 7777120, significantly reduced the levels of MIP-1α, RANTES, IL-4, MCP-1, IL-1β, IL-6, KC, and GM-CSF. TNFα was detected at low levels in the FITC challenged ears and this was significantly reduced by treatment with JNJ 7777120. The inhibition of the cytokine and chemokine levels was only partial, but this was consistent with effects on tissue cytokines seen in lung inflammation models (Dunford et al. 2006). Similar results were seen with the other $H_4R$ antagonist, JNJ 28307474 (data not shown). These results, along with the effect on ear edema at 24 h, clearly support a role for the $H_4R$ in Th2-mediated skin inflammation.

***Ex vivo* antigen re-stimulation is impaired in H4R antagonist dosed mice**

FITC-induced dermatitis in Balb/C mice is suggested to be CD4+ T cell-dependent and Th2-type driven (Dearman and Kimber 2000), and previous work has shown that the $H_4R$ is involved in Th2 cell activation (Dunford et al. 2006). In order to determine if the $H_4R$-mediated inflammation in this model is due to effects on T cell function, Th2 cytokine levels were measured upon *ex vivo* antigen stimulation of lymph nodes. Draining lymph nodes were

harvested after ear edema measurements were taken. The total number of cells in the draining lymph node of FITC challenged mice was not different between vehicle and compound treated mice, but was increased compared to mice whose ears were not challenged with FITC. An equal number of cells were then cultured for 72 h in the presence of FITC and proliferation, as well as cytokine production, was measured. Cells from draining lymph nodes isolated from FITC exposed mice proliferated with *ex vivo* FITC stimulation and this was reduced in cells from the lymph nodes of mice dosed with JNJ 7777120 *in vivo* (Figure 5a). There was no change in proliferation upon anti-CD3/anti-CD28 stimulation suggesting that the effect was antigen specific. After antigen re-stimulation the production of IL-4, IL-5 and IL-17 was inhibited by treatment *in vivo* with JNJ 7777120 (Figures 5b-d). This result suggests that $H_4R$ antagonism impairs Th2 T cell functions and this contributes to the decreases in dermal inflammation in this model.

**Dendritic cells migration is impaired by H4R antagonism**

The above results suggest that $H_4R$ is involved in Th2-dependent inflammatory responses. In order to better understand the mechanisms involved, we tested the possibility that $H_4R$ plays a role in dendritic cell migration, as has been shown *in vitro* for human monocyte-derived dendritic cells and other cell types (Damaj et al. 2007; Gutzmer et al. 2005; Hofstra et al. 2003; Ling et al. 2004). Antigen-bearing dendritic cells were detected as positive for both FITC and two dendritic cell markers - CD11c and MHC II. FITC positive dendritic cells were detected in the auricular lymph nodes after FITC application on the ear (Figure 6a). Lymph nodes from animal dosed with JNJ 7777120 contained a lower number of FITC positive dendritic cells and this was evident with either CD11c or MHC II staining (Figure 6b,c). These results suggest that

the $H_4R$ is involved in the control of the migration of antigen-carrying dendritic cells to draining lymph nodes, and therefore can affect T cell priming.

**Inflammatory Pruritus is inhibited by a $H_4R$ antagonist**

Previously the $H_4R$ has been shown to be involved in mouse models of acute pruritus. Since pruritus is a hallmark feature of many inflammatory skin diseases such as atopic dermatitis, studies were performed to see if $H_4R$ antagonists were anti-pruritic in a disease model. The model (Figure 7a) was modified slightly from that used to assess inflammation (Figure 1a) to yield a stronger itch component. The scratching response in mice was attenuated by pretreatment with JNJ 7777120 in a dose dependent manner (Figure 7b). The $H_1R$ antagonist fexofenadine given at a dose (150 mg/kg) previously shown to completely inhibit histamine-induced edema formation (Dunford et al. 2007), had no significant effect on pruritus alone, nor did it enhance the effect of JNJ 7777120.

As for the previous model (Figure 1a), FITC application in this model led to ear edema when measured 24 h after the challenge. JNJ 7777120 given 20 min prior to and 4 h after FITC application on the ear reduced the ear edema in a dose dependent fashion (Figure 7c). The dose response mimicked that seen with pruritus reduction. Once more, the effect of an $H_1R$ antagonist in this model was studied and fexofenadine (150 mg/kg) did not show any inhibition of ear edema (Figure 7c), nor was there any additive effect when dosed in combination with JNJ 7777120. This indicates that an $H_4R$ antagonist, but not an $H_1R$ antagonist can have both anti-inflammatory and anti-pruritic effects in this model.

To assess whether the effect of the $H_4R$ antagonists was dependent on mast cells, the extended FITC model (Figure 7a) was carried out in mast cell sufficient (WBB6F1 +/+) and mast cell deficient mice (WBBF1 $W/W^v$). FITC application was able to induce edema and scratching in mast cell deficient mice and both could be inhibited by JNJ 7777120 (Figure 7d,e). This indicates that mast cells are not required for the $H_4R$-mediated responses and that they are not the source of histamine for activating the $H_4R$. Interestingly, while the edema was equivalent in both the mast cell deficient and wild-type animals, the scratching was much greater in the mast cell deficient mice. The mechanisms for this are unknown, but it has been seen previously with both substance P and compound 48/80 induced itch, although to a lesser extent (Hossen et al. 2003; Inagaki et al. 2002). Recently, it has been recognized that mast cells can have both negative and positive regulatory functions depending on the physiological situation (Galli, Grimbaldeston, and Tsai 2008).

# Discussion

Histamine has been implicated in the pathophysiology of atopic dermatitis, but antihistamines that target the $H_1R$ are generally not considered to be effective (Akdis et al. 2006). Here I have used a mouse model to test the efficacy of $H_4R$ antagonists against allergic inflammation and pruritus in the skin. FITC challenge is a contact dermatitis model, but it has several features similar to atopic dermatitis in that it is IgE, Th2 cytokine and CD4+ T cell dependent (Dearman and Kimber 2000; Takeshita et al. 2004). In particular the model is characterized by strong eosinophilia that distinguishes it from other Th2 sensitizers (Figure 3 and Takeshita et al. 2004). Here I have confirmed the Th2 nature of this model by showing increases in eosinophils, mast cells and Th2 cytokines in the ear following FITC challenge.

Previous work has suggested that the $H_4R$ modulates allergic lung inflammation mainly through its effects on Th2 cell induction. The data shown here demonstrate that this is also true for Th2 responses in the skin. The levels of several pro-inflammatory cytokines and chemokines including IL-4 were increased in ear tissue upon FITC challenge and were inhibited by treatment with $H_4R$ antagonists. While the effects of dexamethasone on cytokine production were not studied, it has recently been shown that it too can reduce the tissue levels of IL-4 in this model (Boehme et al. 2009). The reduction in these inflammatory mediators may lead directly to an attenuation of edema formation, as it has been previously shown that blocking IL-4 and TNF-α can decrease edema in this model (Takeshita et al. 2004; Suto et al. 2006). In addition the number of mast cells and eosinophils in the ear were increased upon exposure to FITC and the levels of both of these could be reduced by treatment with $H_4R$ antagonists. The effects on mast cells and eosinophils could be indirect due to changes in cytokine or chemokine levels, or could be a direct effect since the $H_4R$ has been shown to mediate chemotaxis for both of these cell

types (Hofstra et al. 2003; Ling et al. 2004). In particular it has been shown *in vivo* that histamine can induce mast cell migration in the trachea and that this can be blocked by an $H_4R$ antagonist (Thurmond et al. 2004).

A direct role for the $H_4R$ on Th2 cell function was seen after antigen restimulation of draining lymph nodes from FITC treated animals. Restimulation with FITC resulted in a profound increase in proliferation of cells from these mice compared to sham animals, which was significantly inhibited in lymph nodes taken from animals dosed with JNJ 7777120. However proliferation in response to anti-CD3/anti-CD28 stimulation was not affected. Similarly stimulation of the lymphocytes with either FITC or anti-CD3/anti-CD28 led to the production of the Th2 cytokines IL-5 and IL-4. The production of these cytokines upon FITC stimulation, but not anti-CD3/anti-CD28 stimulation, was significantly inhibited in lymph nodes taken from animals given JNJ 7777120. In addition, IL-17 was also produced upon antigen restimulation and this was inhibited by *in vivo* treatment with JNJ 7777120. Similar results have been reported in a mouse allergic lung inflammation model (Dunford et al. 2006). These results suggest that as for the lung, the $H_4R$ can modulate Th2 T cell responses in the skin.

In the previous study it was shown that $H_4R$ on dendritic cells was necessary for proper stimulation of Th2 cells in vitro (Dunford et al. 2007). Therefore, some of the effects of $H_4R$ antagonists on *in vivo* Th2 responses may be directly related to the activation of Th2 cells. In addition to this, it appears that the $H_4R$ can mediate migration of dendritic cells from sites of inflammation to the lymph nodes. Dendritic cells and Langerhans cells in the skin are important antigen presenting cells necessary for the activation of T cells and are known, at least in the case

of dendritic cells, to express the $H_4R$. Recently, it has been shown that the $H_4R$ is expressed on human inflammatory dendritic epidermal cells that are found in lesions of atopic dermatitis patients (Dijkstra et al. 2008). After activation, antigen presenting cells migrate from the site of inflammation to the draining lymph nodes where they interact and activate T cells. In this model treatment with an $H_4R$ antagonist reduced the number of FITC+ dendritic cells in the draining lymph nodes. Therefore, the reduction in the number of antigen presenting cells migrating to the lymph node with $H_4R$ antagonism may contribute to a reduction in Th2 cell activation.

The effect on the number of dendritic cells in the lymph nodes may be an indirect effect related to the reduction in tissue cytokines and chemokines by $H_4R$ antagonism. In particular, TNF-a, IL-1B, GM-CSF and MCP-1 have all been shown to mediate dendritic cell or Langerhans cell migration from the skin to the lymph node (Cumberbatch and Kimber 1995; Cumberbatch et al. 2000; Cumberbatch, Dearman, and Kimber 1997; Cumberbatch et al. 1999; Cumberbatch et al. 2003; Suto et al. 2006; Smith et al. 1998; Mizumoto et al. 2001). In addition to the potential indirect effect, histamine acting via the $H_4R$ directly on human monocytes-derived dendritic cells or mouse bone-marrow derived dendritic cells has been shown to induce chemotaxis in vitro (Damaj et al. 2007; Gutzmer et al. 2005; Bäumer et al. 2008). Furthermore, Bäumer et al. have recently shown that histamine can enhance dendritic cell migration from mouse ear explants and that this effect could be blocked by JNJ 7777120 (Bäumer et al. 2008). Therefore, histamine can directly induce chemotaxis or can prime dendritic cells for activation by other chemokines to promote migration to the lymph nodes. Decreased dendritic cell migration to the lymph nodes should lead to reduced activation of T cells consistent with the reduction in T cells in the tissue and cytokine production.

The effects of the $H_4R$ antagonists on Th2 cytokines appeared to translate into a reduction in ear edema upon application of FITC. The inhibitory effect on edema is clearly $H_4R$ mediated since two chemically distinct compounds show equivalent effects and similar effects are seen in $H_4R$-deficient mice. However this reduction was only partial, indicating that only a portion of the edema is $H_4R$ mediated. This is consistent with a previous report showing that blocking the T cell cytokines IL-4 or IL-5 in this FITC model only gives a partial reduction in ear edema at 24 h (Takeshita et al. 2004). Interestingly, the inhibition seen with either $H_4R$ antagonist was equivalent to that seen with dexamethasone.

In addition to the anti-inflammatory effects of $H_4R$ antagonists, this study shows that the compounds were also anti-pruritic in this mouse model of allergic skin inflammation. Histamine has long been known to be a mediator of itch in normal human skin and it induces increased pruritic responses in diseased skin of atopic dermatitis patients compared to normal skin (Steinhoff et al. 2003). However, the role of histamine in the pruritus associated with atopic dermatitis is much less clear mainly due to the fact that $H_1R$ antihistamines are generally considered to be ineffective in the treatment of atopic dermatitis-associated pruritus (Klein and Clark 1999; Akdis et al. 2006). Previous work has shown that the $H_4R$ receptor is involved in acute pruritus in mice induced by histamine, mast cell degranulation or direct stimulation of neurons (Dunford et al. 2007). In the model presented here the reduction in pruritic responses may due to a reduction in inflammation or a direct effect on sensory neurons, as postulated for the acute pruritus models.

It is of interest that both the edema and the pruritic responses to FITC are retained in the $W/W^v$ mice and that the $H_4R$ antagonist was still able to block both responses. This suggests that mast cells are not required for either the $H_4R$-mediated edema or pruritic responses, although care should be taken with this interpretation since these mice still have some skin mast cells (<1% of the wild-type levels) and have other defects including a slight neutropenia that may affect the response (Nigrovic et al. 2008; Tsai et al. 2005). The mast cell-independent effects of the $H_4R$ on the edema and pruritus in this model is consistent with previous data in a mouse asthma model and other pruritus models (Dunford et al. 2007; Dunford et al. 2006). However, the mechanism for development of pruritus immediately after the application of FITC in the absence of mast cells in unknown. It is possible that it triggers histamine or other mediator production from other cells such as dendritic cells or keratinocytes. Intriguingly it was recently shown that the $H_4R$ can mediate the production of IL-31 (Gutzmer et al. 2009) and this cytokine has been linked to pruritus in atopic dermatitis (Castellani et al. 2006; Dillon et al. 2004). In addition the data suggests that mast cells are not the source of histamine that activates the $H_4R$. Several other cell types in the skin have been shown to have the capacity to produce histamine upon stimulation including dendritic cells and keratinocytes (Malaviya, Morrison, and Pentland 1996; Dunford et al. 2006).

Here it is shown that an $H_1R$ antagonist, fexofenadine, is not effective against either the inflammatory or pruritic responses in this model. The lack of effect on pruritus is consistent with the previous findings in acute pruritus models (Dunford et al. 2007) and with the lack of effect of

second-generation antihistamines, including fexofenadine, on itch in atopic dermatitis patients (Klein and Clark 1999). Furthermore, the fact that the $H_1R$ antagonist cannot block the FITC-induced edema indicates that histamine itself is probably not driving this response, since this dose of fexofenadine can completely inhibit histamine-induced edema (Dunford et al. 2007). However, these conclusions should be taken with caution since they may depend on the $H_1R$ antagonist used. For example, diphenhydramine has been shown to block histamine and antigen-induced itch, which is thought to be due to its central activity (Rossbach et al. 2009; Dunford et al. 2007). It has also been shown that loratadine can inhibit histamine-induced itch whereas fexofenadine does not, however neither appear to inhibit compound 48/80 induce scratching (Hossen et al. 2005; Dunford et al. 2007). Whether these differences have to do with differences in distribution such as CNS penetration or perhaps non-$H_1R$ related effects of the compounds is not known.

Concurrent with this research another study appeared addressing the effects on JNJ 7777120 on the pruritus induced by two other haptens, 2,4-dinitrochlorobenzene and toluene-2,4-diisocyanate (Rossbach et al. 2009). Consistent with the data shown in this work, JNJ 7777120 was able to significantly inhibit the pruritus induced by either hapten. However, the edema formation 24 h after hapten challenge was not affected. The difference between my finding and those reported in this work concerning the anti-inflammatory properties of $H_4R$ antagonists are unclear, but may reflect differences in mouse strains used or in the mechanism of action of the haptens. This is especially true for 2,4-dinitrochlorobenzene that is described as inducing a Th1-dependent effect (Rossbach et al. 2009). In particular the FITC model used here has a strong eosinophil component that is not found in other contact dermatitis models (Figure 3 and Takeshita et al.

2004). Eosinophilic inflammation may be particularly sensitive to $H_4R$ antagonism since it has been shown that eosinophil chemotaxis can be directly mediated by the $H_4R$ (Ling et al. 2004; Buckland, Williams, and Conroy 2003) and reductions in eosinophils have also been seen in asthma models (Dunford et al. 2006). The results presented here are also consistent with the effects of thioperamide, a dual $H_3R/H_4R$ antagonist, in reducing edema and eosinophil infiltration in another skin inflammation model (Hirasawa et al. 2009).

The data presented in this study show an effective anti-pruritic and anti-inflammation function of $H_4R$ antagonists in a mouse model of Th2-dependent skin inflammation. This effect was superior to that of $H_1R$ antagonists. The anti-inflammatory properties appeared to be driven by a reduction in Th2 cell activation that can partially be accounted for by a reduction in the migration of antigen-bearing dendritic cells to the lymph nodes. Therefore, the effects of the $H_4R$ antagonists on pruritus, inflammation and Th2 cell responses point to their therapeutic potential for the treatment of inflammatory skin disorders such as atopic dermatitis.

# Part 2 $H_4R$ and Rheumatoid Arthritis

# Abstract

The histamine $H_4$ receptor ($H_4R$) has been shown to drive inflammatory responses in models of asthma, colitis and dermatitis and in these models it appears to impact both innate and adaptive immune responses. In this study I utilized both $H_4R$-deficient mice and a specific $H_4R$ antagonist, JNJ 28307474, to investigate the involvement of the $H_4R$ in mouse arthritis models.

$H_4R$-deficient mice and wild-type mice administered the $H_4R$ antagonist were studied in models of collagen antibody-induced arthritis (CAIA) and collagen-induced arthritis (CIA). The impact on Th17 cells was assessed by restimulation of inguinal lymphocytes in the disease or immunization models and with*in vitro* stimulation of whole blood.

Both $H_4R$-deficient mice and mice treated with the $H_4R$ antagonist exhibited reduced arthritis disease severity in both CAIA and CIA models. This was evident from the reduction in disease score and in joint histology. In the CIA model treatment with the $H_4R$ antagonist reduced the number of IL-17 positive cells in the lymph node and the total production of IL-17. Th17 cell development *in vivo* was reduced in $H_4R$-deficient mice or in mice treated with an $H_4R$ antagonist. Finally, in both mouse and human treatment of blood with an $H_4R$ antagonist reduced the production of IL-17 when cells were stimulated *in vitro*.

These results implicate the $H_4R$ in disease progression in arthritis and in the production of IL-17 from Th17 cells. This work supports future clinical exploration of $H_4R$ antagonists for the treatment of rheumatoid arthritis.

# Introduction

The histamine $H_4$ receptor ($H_4R$) has been linked to inflammation in several preclinical models and it holds promise as a target for treating allergic inflammation (for recent review see Walter, Kottke, and Stark 2011). Not so obvious would be a role in autoimmune diseases, although changes in histamine levels have been observed in such conditions (Tuomisto, Kilpelainen, and Riekkinen 1983; Frewin et al. 1986; Winterkamp et al. 2002). In addition $H_4R$ expression has been found in the synovial cells, primarily on fibroblast-like and macrophage-like cells, from rheumatoid arthritis patients (Ohki et al. 2007; Ikawa et al. 2005).

Most of the models showing a role for the $H_4R$ in inflammation are allergic or Th2-driven inflammation that is commonly associated with histamine involvement. However, the $H_4R$ has been shown to mediate T cells responses in humans and mice (Dunford et al. 2006; Cowden, Riley, et al. 2010; Cowden, Zhang, et al. 2010; Lundberg et al. 2011; Gutzmer et al. 2009). The effects on T cells has prompted the question as to whether the $H_4R$ has roles beyond Th2 driven inflammation and whether the receptor could be involved in autoimmune diseases (Zhang, Venable, and Thurmond 2006). The receptor has been shown to be expressed on human Th17 cells and in these cells can mediate the production of IL-17 (Mommert et al. 2012). Consistent with this, $H_4R$-dependent decreases in IL-17 have been consistently shown even in mouse Th2 driven inflammation models (Dunford et al. 2006; Cowden, Riley, et al. 2010; Cowden, Zhang, et al. 2010).

In this work the requirement for the $H_4R$ is shown in both a mouse collagen-induced and a collagen antibody-induce arthritis model. Having effects in both models suggest a role for the

$H_4R$ in both innate and adaptive immune responses that drive arthritis in humans. In particular, one of the underlying mechanisms for the $H_4R$ effects may be in part due to modulation of Th17 cells. These results suggest that antagonism of the $H_4R$ is a promising target to treat autoimmune diseases such as rheumatoid arthritis.

# Materials and methods

**Arthritis Models**

For the collagen antibody-induced arthritis (CAIA) model BALB/c mice were given 2 mg collagen antibody cocktail (Chondrex, Inc., Redmond, WA) intravenously on day 1 and then challenged with 20 $\mu$ g LPS by intraperitoneal injection (ip) on day 3. Disease onset occurred on day 4, and mice were examined visually daily for the appearance of arthritis in the peripheral joints. For the collagen-induced arthritis (CIA) model DBA1/J mice were injected at the base of the tail with bovine type II collagen (Chondrex, Inc., Redmond, WA) emulsified in complete Freund's adjuvant (CFA) per the manufacture's protocol. On day 26 mice received 20 µg of LPS ip to synchronize the onset of arthritis. Animals were enrolled into treatment groups on days 27-28 when any paw had a score of 1 or greater. To induce arthritis in C57BL/6 $H_4R$ deficient and wild-type animals the method was modified to included two CFA/collagen injection similar to that described previously (Inglis et al. 2008). For all models the severity of arthritis was graded on a scale of 0–4 for each paw in a blinded fashion. The scores for each of the four paws were added together to give a final score such that the maximal severity score was 16 and are presented as Mean±SEM. Where applicable, mice were treated orally (by gavage) with vehicle or the $H_4R$ antagonist JNJ 28307474 at the indicated doses twice a day at the time of disease onset (defined as a score of 1 or greater in any paw). Paw tissue was prepared and histological analyses were performed as previously described (Bendele et al. 2000). In addition inguinal lymph nodes were collected in some studies and pooled per treatment group. A single cell suspension (RPMI 1640 supplemented with 10% fetal bovine serum, nonessential amino acids and 2-β-mercaptoethanol) was prepared and triplicates ($10^5$ cells/well) were plated in 96 well

plate coated with 2 µg/ml of anti-CD3 and 1 µg/ml anti-CD28. After 24 h supernatants were collected and IL-17 and IFNγ were measured by ELISA. In addition cell were stained for CD4 and intracellular IL-17 and analyzed by FACS.

**Th17 Cells Models**

An adoptive transfer model for Th17 cell development was previously described (McGeachy et al. 2009). Where indicated mice were treated with vehicle or JNJ 28307474 (50 mg/kg twice daily) starting the day after transfer of the OT-II cells just prior to the immunization.

**Statistical Analysis**

Details on the statistical analysis are given in each figure caption. All statistical analysis was carried out using GraphPad Prism (San Diego, CA).

# Results

**Collagen Antibody-induced Arthritis (CAIA) Model**

Wild-type and $H_4R$-deficient mice on the BALB/c background were studied in the CAIA model. In wild-type mice there is an increase in clinical score that peaks and plateaus around Day 5 (Figure 8a). The same pattern is seen in the $H_4R$-deficient mice, but the disease severity as judged by the clinical scores is dramatically decreased. A Wilcoxon Ranked Sum test indicated a statistical difference in the time courses ($p < 0.01$) and there was a statistical significant difference between wild-type and $H_4R$-deficient mice at every time point. When the disease severity is expressed as area under the curve (AUC), a statistically significant reduction in disease severity can be clearly visualized (Figure 8b). Histological examination was conducted to illustrate the joint pathology in diseased mice (Figure 9a). Consistent with the clinical score the $H_4R$-deficient mice had a significant reduction in disease pathological severity was observed as indicated by inflammation, pannus, cartilage damage and bone damage (Figure 9b). As mast cells are a potential source of histamine in the synovium, the numbers of mast cells along the inflamed synovial lining of the diseased mice in different fields were counted, and a significant reduction of the mean mast cell numbers were observed between the wild-type and $H_4R$-deficient mice.

The data with the $H_4R$-deficient mice point to a role for the receptor in mediating the inflammation seen in this model. To confirm this JNJ 28307474, a potent and specific $H_4R$ antagonist (Cowden, Zhang, et al. 2010) with a relatively long half-life in mice (Cowden, Yu, Challapalli, Huang, Kim, Fung-Leung, Ma, Riley, Zhang, Dunford Paul, et al. 2013), was used. JNJ 28307474 was given orally at various doses twice a day starting at the time of disease onset

(any paw with a score of 1 or greater). Treatment with 20 and 50 mg/kg JNJ 28307474 significantly reduced the severity score as seen both from the time course ($p<0.01$ for 50 mg/kg by Friedman test) and the AUC of the score (Figure 10 a and b). The inhibition observed at 50 mg/kg was similar to that seen in the $H_4R$-deficient mice (Figure 8 b).

The $H_4R$ is expressed on several cell types that may be involved in modulating the inflammation in this model. Of particular interest are dendritic cells since previously it was shown that lack of the $H_4R$ on splenic $CD11c^+$ cells impaired their ability to activate T cells (Dunford et al. 2007). To investigate the role of these cells in the CAIA model, $CD11c^+$ cells isolated from the spleens of wild-type or $H_4R$-deficient mice were injected into $H_4R$-deficient mice before antibody administration. $H_4R$-deficient mice that received $H_4R$-deficient $CD11c^+$ cells had a reduced severity score compared to wild-type mice. However, when these mice received wild-type $CD11c^+$ cells the severity score was similar to wild-type mice, suggesting that the $H_4R$ on these cells was contributing to the disease progression.

**Collagen-induced Arthritis (CIA) Model**

To further understand the role of the $H_4R$ in mediating arthritis, a collagen-induced arthritis model was used. As for the CAIA model, treatment with JNJ 28307474 led to a dose-dependent reduction in the disease severity score with the highest dose of 50 mg/kg showing little increase in disease activity over the baseline (Figure 11a). The time courses for the 20 and 50 mg/kg doses were statistically reduced compared to the vehicle control ($p<0.001$ with a Friedman test). Calculation of the AUC of the severity score indicated that there was a trend for reduction at 5 and 20 mg/kg, but a statistically significant reduction at 50 mg/kg (Figure 11b).

The reduction in severity score with the $H_4R$ antagonist suggests that the receptor mediates inflammation in this model. To confirm this, studies were carried out in $H_4R$-deficient mice. However, the standard model is conducted in DBA1/J mice and the $H_4R$-deficient mice were on the C57BL/6 background and therefore, the model was adapted to this strain (Inglis et al. 2008). Prior to receiving the LPS boost the incidence of arthritis in the $H_4R$-deficient mice was lower (1/12) than in wild-type mice (10/12) and the average score was significantly reduced (Figure 11c). After receiving the LPS boost all of the wild-type animals (12/12) developed scores of greater than 2, but only 7 of 12 $H_4R$-deficient animals developed disease. At this point there was no statistically significant difference in the disease scores (Figure 11c). Fourteen days later, all of the wild-type mice still exhibited the same level of disease as at Day 5, but the disease appeared to improve in the $H_4R$-deficient mice as evidenced by two mice (final incidence 5/12) completely recovering (score <2) and a statically significant decrease in the average score starting at Day 9 (Figure 11c). Furthermore, if wild-type mice were treated with JNJ 28307474 on Day 5 after they all develop disease, the mice start to recover as measured by a decrease in the average disease score (Figure 11c) and at Day 19 the average disease score is similar to that seen in the $H_4R$-deficient mice. Overall there was a statistically significant decrease in the incidence of arthritis in the $H_4R$-deficient mice using either a Fisher's Exact test ($p < 0.007$) or a log-rank survival method ($p < 0.002$). In total these results confirm that the $H_4R$ can mediate inflammation in the mouse CIA model.

Histological examination was also conducted in this model to illustrate the joint pathology in diseased mice and representative data is shown in Figure 12a. Scoring of inflammation, pannus,

cartilage damage and bone damage showed inhibition by 50 mg/kg JNJ 28307474 for all of these parameters (Figure 12b). This is consistent with the effects seen with the CAIA model. As for the CAIA model, the mast cell numbers in the joint were decreased with treatment with JNJ 28307474 and these have been reported to be increased in the model (Kakizoe et al. 1999). To further support the histological finding of a reduction in joint inflammation, the expression of inflammatory cytokines and chemokine in the joint was assessed. The message levels of IL-6. IL-1α, MCP-1, MIP-2 and TNF were all reduced in the joints of animals treated with JNJ 28307474, although only the reductions in IL-6, MCP-1, MIP-2 reached statistical significance. There was not a statistically significant reduction in collagen specific IgG levels.

**Th17 Cell Development**

In the CIA model the percentage of IL-$17^{+}CD4^{+}$ cells in the inguinal lymph node were increased in diseased animals compared to naïve animals and treatment with JNJ 28307474 lead to a reduction in this percentage (Figure 13a). In addition restimulation of lymphocytes with anti-CD3 and anti-CD28 resulted in the production of IL-17 in diseased animals and this was reduced when the animals were treated with JNJ 28307474 (Figure 13b). No effect of the $H_4R$ antagonist on IFNɤ was seen, although it was increased in diseased animals (Figure 13b).

It is difficult to determine whether the reduction in Th17 cells seen with $H_4R$ antagonist treatment in CIA is due to a direct role of the $H_4R$ in Th17 cell function or whether this only reflects a reduction in the inflammation driven by other anti-inflammatory mechanisms. Therefore, the role of the $H_4R$ on Th17 cell development *in vivo* was directly assessed using an adoptive transfer model with transgenic OT-II T cells specific for ovalbumin (McGeachy et al.

2009). Treatment with JNJ 28307474 led to a reduction in the number of OT-II $Th17^+$ cells in the lymph node (Figure 13c). A role for the $H_4R$ in Th17 cell development *in vivo* was confirmed using $H_4R$-deficient mice. Transferring $H_4R$-deficient OT-II cells into wild-type or $H_4R$-deficient mice also led to the reduction in the total number of OT-II $Th17^+$ cells as seen with $H_4R$ antagonist treatment (Figure 13d). In addition transfer of wild-type OT-II cells into $H_4R$-deficient mice also produced the same effect. These results show that the $H_4R$ on both T cells and other host cells are necessary for Th17 cell development *in vivo*.

Recently, $H_4R$ expression has been shown on human Th17 cells and the production of IL-17 is increased by treatment with an $H_4R$ agonist (Mommert et al. 2012). Mouse Th17 cells can also express the $H_4R$ as determined by RT-PCR (data not shown) and the impact of the $H_4R$ on IL-17 by these cells was explored. Blood stimulated with anti-CD3/CD28 and IL-23 led to an increase in IL-17 production and this was decreased in blood taken from $H_4R$-deficient mice or mice treated in vivo with the $H_4R$ antagonist JNJ 7777120 (Figure 13e).

A similar effect on IL-17 production can be seen with human cells. A variety of stimuli were able to induce IL-17 production from human PBMC with the highest levels produced when a combination of anti-CD3, anti-CD28, IL-23 and IL-1β were used (Figure 13f). Treatment *in vitro* with either JNJ 7777120 or JNJ 28307474 was able to reduce the IL-17 level under all stimulation conditions. These results show that in humans and mice, the $H_4R$ can directly modulate IL-17 production.

# Discussion

Previously the $H_4R$ has been shown to play a role in Th2 driven allergic models (Dunford et al. 2006; Cowden, Riley, et al. 2010; Cowden, Zhang, et al. 2010). The effect in these models was postulated to be driven by inhibition of T cell responses. Indeed in the mouse asthma model, mice treated with an $H_4R$ antagonist only during the sensitization phase of the model, where T cell responses are initiated, exhibit reduced disease (Dunford et al. 2006; Beermann et al. 2012). This effect on T cells prompted the question as to whether the $H_4R$ could modulate responses of other T cell subtypes and, therefore, have a role in autoimmune diseases.

The data presented here supports a role for the $H_4R$ in arthritis. In a CAIA model, $H_4R$-deficient mice were largely protected from disease as judged by a reduction in disease score and by joint histology. A very similar effect was seen when mice were treated with the $H_4R$ antagonist, JNJ 28307474. The fact that there are similar effects with $H_4R$-deficient mice as with an $H_4R$ antagonist strongly supports a role for the $H_4R$ in this model. These results are similar to those seen in the K/BxN model of arthritis with histidine decarboxylase deficient mice that lack histamine (Rajasekaran et al. 2009). The CAIA and K/BxN transfer model have similar underlying mechanisms and, therefore it is reasonable to assume that the effects reported in histidine decarboxylase deficient mice are due to lack of histamine activation of the $H_4R$. Both of these models are thought to be driven by the activation of the innate immune system and T cells are not thought to be involved until later in the disease progression. While the exact mechanisms for the role of the $H_4R$ in the models are not known, transfer of wild-type $CD11c^+$ cells can restore the disease in $H_4R$-deficient mice and there is evidence that the receptor can play a role in mast cell, dendritic cell, NK T cell and macrophage activation (Dunford et al. 2006; Desai and

Thurmond 2011; 2013; Leite-de-Moraes et al. 2009). Therefore, accumulating data suggests that $H_4R$ is a crucial player in modulating innate cell activation that is importing for initiating inflammatory responses and explains the effects seen in the CAIA model.

To further explore the potential role of the $H_4R$ in arthritis a CIA model was employed that has a strong T cell component. As for the inflammatory arthritis model, both $H_4R$-deficient mice and mice treated with an $H_4R$ antagonist, JNJ 28307474, exhibited a reduction in severity score and inflammation. $H_4R$ antagonist treatment is effective whether the compound is given semi-therapeutically after the mice show the first signs of disease (Figure 11a) or therapeutically when animals have the maximum score (Figure 11c). Similar effects where observed in $H_4R$-deficient mice. Interestingly prior to the LPS boost, $H_4R$-deficient mice had a lower incidence of disease compared to wild-type mice. The administration of LPS led to increased disease in both the wild-type and $H_4R$-deficient animals, although the incidence and average score trended to be lower in the $H_4R$-deficient mice. So it appears that the $H_4R$-deficient mice are protected from developing arthritis in the model, but that some of this can be overcome by adding a strong inflammatory stimuli such as LPS. However, even though the LPS initially tended to increase the disease score in the $H_4R$-deficient mice, after this point they start to recover whereas the wild-type mice have stable disease. Consistent with this, wild-type mice treated with JNJ 28307474 after the LPS boost, when they have the maximum score, start to recover and have a similar severity score to the $H_4R$-deficient mice on day 19 that is significantly better than that of the wild-type mice.

The effects of the $H_4R$ in the CIA model could be at least partly mediated by effects on Th17 cells. It is known that the model is dependent on Th17 cells (Lubberts et al. 2004; Nakae et al.

2003; Lubberts et al. 2001) and treatment with the $H_4R$ antagonist significantly reduced the number of IL-$17^+$ cells in the lymph node and the secretion of IL-17 when lymphoctyes were stimulated *ex vivo*. This could result from a direct role for the $H_4R$ in Th17 cell function since in an immunization model, $H_4R$-deficient mice or mice treated with an $H_4R$ antagonist had a reduction in the development of Th17 cells (Figure 13 c,d). Of interest, the effects on the development of Th17 cells in the adoptive transfer model were apparent when either the donor T cells or the recipients were $H_4R$-deficient. This implies that the receptor is required on both T cells and antigen presenting cells for optimal Th17 cell development. The results in mice appear to be consistent with effects on human Th17 cells, where blocking the $H_4R$ *in vitro* inhibits IL-17 production from human PBMC. This is consistent with recent work showing that human Th17 cells express the $H_4R$ and that IL-17 production can be increased with an $H_4R$ agonist (Mommert et al. 2012). Therefore, the $H_4R$ appears to play a direct role in Th17 activity and may explain the effects in the CIA model, although other mechanisms such as effects on macrophages and NK T cells may be involved.

One outstanding question is the source of histamine in the animal models and its relevance to human arthritis. It is well-known that mast cells and basophils secrete histamine and thus are potential sources. Mast cells are known to be increased in the CIA model (Kakizoe et al. 1999) and here it is shown that treatment with an $H_4R$ antagonist in both models reduces the number of mast cells in the synovial lining. Mast cells have been shown to be important mediators in some animal models of arthritis and have been found to be increase in the synovium of rheumatoid arthritis patients; however it is still not clear whether they are key players in the disease (for reviews see Nigrovic and Lee 2007; Suurmond, Schuerwegh, and Toes 2010). Basophils may

also play a role and it was recently shown that histamine release from basophils amplifies IL-17 release from T cells (Wakahara et al. 2012). Finally, it has become apparent that many immune cells such as dendritic cells, T cells and neutrophils are capable of producing histamine when stimulated (Dunford et al. 2006; Alcaniz et al. 2013; Aoi et al. 1989). It has been speculated that local production of histamine by dendritic cells can act in an autocrine fashion to modulate dendritic cell/T cell interactions (Thurmond, Gelfand, and Dunford 2008) and this local production of histamine in the joint or at sites of T cell activation may be the most relevant for $H_4R$ activation in human arthritis.

Overall the data presented show an anti-inflammatory role for $H_4R$ antagonist in preclinical models and support the clinical study of such antagonist for the treatment of rheumatoid arthritis. In addition, the known safety profile of the $H_4R$ also supports clinical testing. The $H_4R$-deficient mice are fertile and healthy and, outside of effects on inflammatory response, appear to have no other defects. In addition no safety issues have been observed with compound treatment either in this work or in other animal models (Cowden, Yu, Challapalli, Huang, Kim, Fung-Leung, Ma, Riley, Zhang, Dunford, et al. 2013; Cowden, Zhang, et al. 2010; Cowden, Riley, et al. 2010; Dunford et al. 2006; Varga et al. 2005). While it is still early, there have been reports of $H_4R$ in phase 1 clinical studies with no safety issues reported (for summary see Salcedo, Pontes, and Merlos 2013). Therefore, $H_4R$ antagonist may provide a safe and effective alternative for the treatment of rheumatoid arthritis.

The work presented here clearly supports a role for the $H_4R$ in arthritis. Importantly, the receptor has effects in both a model of inflammatory arthritis and one of autoimmune arthritis suggesting

that it can impact both innate and adaptive immune responses. There are several potential mechanisms underlying this role including possible effects on Th17 cells. Taken as a whole, the current data suggests that the $H_4R$ can be viewed not necessarily as the initiator of inflammation, but as a potentiator of inflammatory responses. This is evidenced by the partial inhibition of TLR-mediated cytokine production previously observed (Dunford et al. 2006; Desai and Thurmond 2011; Cowden, Yu, Challapalli, Huang, Kim, Fung-Leung, Ma, Riley, Zhang, Dunford Paul, et al. 2013) and the fact that LPS can cause a flair in severity score in the $H_4R$-deficient mice that then resolves compared to wild-type mice. Therefore, antagonist of the receptor would not be expected to be immunosuppressive, but rather lead to a dampening of the initial inflammatory response and thereby leading to a reduction in inflammation in a variety of disease states, be they allergic or autoimmune in nature.

# General Discussion

Although histamine, a biologically active amine, has been known for nearly 100 years its role and function are still being discovered and understood. For much of that time only two histamine receptors $H_1R$ and $H_2R$, were studied or known. Around 2000, two new receptors for histamine were discovered, $H_3R$ and $H_4R$. Their role and function was completely unknown at the time. Many scientists including myself began research to understand what if any function these new receptors played in disease. $H_3R$ was expressed in the central nervous system as was the focus of other researchers. However, in addition to expression in the central nervous system, $H_4R$ was expressed on immune cells. As an immunologist this was a unique opportunity determine the function of this receptor. Also key tools like knock out mice and receptor antagonists were generated that enabled me to understand the consequence of inhibiting $H_4R$. In this thesis I detailed the role of $H_4R$ in atopic dermatitis and Rheumatoid Arthritis although it should be noted others and myself have reported on its role in asthma, colitis, and pain as well.

In part one; I learned $H_4R$ does play a key role in AD. Specifically, $H_4R$ reduces the amount of inflammation in the skin in pre-clinical model of AD. FITC, a chemical was applied to mice to generate inflammation in the skin and the animals were treated with $H_4R$ antagonist. $H_4R$ antagonist reduced inflammation as measured by the ear skin thickness after FITC application. It reduces numbers of mast cells and eosinophils found in the skin characterized by histology. To understand why there were fewer eosinophils and mast cells I began to look at the adaptive immune response. First area of study was understanding the role of $H_4R$ antagonist on dendritic cell (DC) migration. Animals treated with $H_4R$ antagonist had fewer dendritic cells in the lymph node after inflammation was induced in the skin. DCs are key gate keepers of the immune system and present antigen to T cells. A decrease in the number of DCs means fewer T cell are activated. This was confirmed by measuring the response of Tcells to antigen ex-vivo

of animals treat with $H_4R$ antagonist. Tcells produce inflammation inducing cytokines such the TH2 cytokines IL-4 and IL-13 that drive the recruitment of mast cell and eosinophil to the skin in AD. However, Tcells from animals treated with $H_4R$ produced reduced amounts of TH2 cytokines IL-4 and IL-13. This results in fewer eosinophils and mast cells being recruited to the skin and less inflammation and disease. This in one key understanding gained from my works.

Another key comorbidity of AD is pruritus. To understand if $H_4R$ played a role in pruritus, I quantified the number of times the animals itched in the same pre-clinical model. Treatment with $H_4R$ antagonist greatly reduced the number of times animals itch as a result of the FITC application. Interestingly, only $H_4R$ antagonist the can penetrate into the central nervous system inhibited this itch and antagonist that are restricted to the periphery do not. Although the key signaling mechanisms still need to discovered, this is the only documented role of $H_4R$ in the central nervous system.

Most important to my research was if these pre-clinical studies would translate into a benefit in patients with AD. Fortunately in the following years other researchers tested $H_4R$ antagonist in AD patients. As predicted by my research AD patients had reduced inflammation in the skin and reported a reduced desire to itch (Murata et al. 2015).

In part two, my research focused on the role of $H_4R$ in Rheumatoid Arthritis (RA). It had been observed that in addition to TH2 cytokine being reduced in the AD studies, another cytokine IL-17 was also reduced. Although IL-17 has little to do with AD it's one of the key cytokines that drive autoimmunity in diseases like RA. So to determine if $H_4R$ antagonist go reduced inflammation in RA I tested two pre-clinical models of RA with the focus on IL-17 levels. The first model, collagen antibody induced arthritis, it’s induced by giving anti-collagen antibodies to

mice. $H_4R$ KO and compound treated animals had reduced inflammation in the joints when quantified with a scoring system. In second model collagen induced arthritis, the results were also the same. Using histology method, the joints were imaged and the inflammation scored. $H_4R$ treated mice had reduced inflammation and joint damage. Critically, the bone and cartilage were protected in mice that received treatment. Next the effect on IL-17 production was quantified. T cells from animals treated with $H_4R$ antagonist produced less IL-17 as measured by FACS and when restimulated *ex vivo*. This reduction in IL-17 is one mechanism of $H_4R$ antagonism but there may be additional mechanism at work as well that have not been uncovered.

Unlike AD, where these preclinical results will apply to human disease, the effect of $H_4R$ on RA patients is yet to be fully understood. Since my work two clinical trials in RA patients with $H_4R$ antagonist have been completed. In the first trial, treatment reduced inflammation and disease severity as expected from my pre-clinical work (Thurmond et al. 2016). However, in second trial there was no effect although the investigators used a lower dose. Until further clinical trials are conducted the role $H_4R$ in human RA is yet to be determined.

In summary, $H_4R$ plays an important role in the immune system as a regulator of TH2 and TH17 inflammation. Antagonism of $H_4R$ lowered TH2 and TH17 driven inflammation resulting in a reduced severity of AD and RA pre-clinically. The result of my research lead to testing of $H_4R$ antagonist in human patients with positive results for AD and undetermined results in RA.

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# Tables

**Table 1. In vitro Ki for JNJ 28307474 Histamine Receptor Binding**

| Receptor | Species | $K_i$ (nM)[1] |
|---|---|---|
| $H_4$ | Human | 4.9 ± 1.1 |
| $H_4$ | Mouse | 109 ± 8 |
| $H_4$ | Rat | 87 ± 9 |
| $H_4$ | Dog | 62 ± 31 |
| $H_4$ | Guinea Pig | 3.4 ± 0.6 |
| $H_3$ | Human | 159 |
| $H_3$ | Rat | 630 |
| $H_1$ | Human | 2501 |
| $H_1$ | Mouse | 1224 ± 208 |
| $H_1$ | Guinea Pig | 3050 |
| $H_2$ | Human | >1000 |

[1] Data given as ± SEM if the assay was run at least two times.

. Table 2. JN28307474 selective by radioligand assay

| Target | % Inhibition at 1µ | Target | % Inhibition At @ 1µ |
|---|---|---|---|
| A1 (h) | - | NK2 (h) | - |
| A2A (h) | 30 | NK3 (h) | - |
| A3 (h) | - | NPY1 (h) | - |
| Alpha 1 | - | NPY2 (h) | - |
| Alpha 2 | 36 | NT1 (h) | - |
| Beta 1 (h) | - | DOP (h) | 10 |
| NE Transporter (h) | 17 | KOP (h) | 28 |
| AT1 (h) | - | MOP (h) | 41 |
| BZD | - | ORL1 (h) | - |
| B2 (h) | - | 5 HT1a (h) | 31 |
| CCKA (h) | - | 5-HT2a (h) | 74 |
| D1 (h) | 36 | 5-HT3 (h) | - |
| D2 (h) | 11 | 5-HT5a (h) | - |
| DA transporter (h) | 24 | 5-HT6 (h) | 21 |
| ETA (h) | - | 5-HT7 (h) | - |
| GABA | - | Sst | - |
| GAL2 (h) | - | VIP1 (h) | - |
| IL-1B (h) | - | VIA (h) | - |
| CCR2 (h) | - | Ca2+ channel | 20 |
| $H_1R$ | - | K+V channel | - |
| $H_2R$ | 42 | SK+Ca channel | - |
| MC4 (h) | 15 | Na + channel | 59 |
| MTI | - | CL- channel | - |

Values are average from three replicates an "-"indicates average percent inhibition less than 10%:

Table 3. In vitro Ki values for JNJ28307474 for various receptors

| Receptor[1] | Ki (nM) |
|---|---|
| 5-HT1b | 500 |
| 5-HT1d | 1600 |
| 5-HT2a | 630 |
| 5HT-2b | 1400 |
| 5-HT2c | 5000 |
| 5-HT7 (rat) | >10000 |
| M1 | 235 |
| M2 | 180 |
| M3 | 100 |
| M4 | 200 |

[1] All receptors were human except otherwise noted

# Figures

**Figure 1. $H_4R$ antagonism reduces dermal inflammation.** *a*, Balb/c mice (n = 7-14 mice per group) were sensitized to FITC on days 0 and 1 and then challenged on day six by application of FITC to one ear. On day seven the difference in ear thickness between the challenged and unchallenged ear was measured with calipers. *b*, The $H_4R$ antagonist, JNJ 7777120, given p.o. 20 min prior to and 4 h after FITC application reduced swelling in a dose dependent manner. The degree of inhibition was similar to that of dexamethasone (Dex) given p.o. at 3 mg/kg. *c*, Ear edema was reduced in $H_4R$-deficient mice ($H_4R$ (-/-)) compared to wild-type (WT) mice. *d*, Ear edema was also inhibited by a chemically distinct $H_4R$ antagonist, JNJ 28307474, given p.o. 20 min prior to and 4 h after FITC application. As for JNJ 7777120, the degree of inhibition by JNJ 28307474 was similar to that of dexamethasone (Dex) given p.o. at 3 mg/kg. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ by one-way ANOVA with post-hoc Bonferroni's test compared to vehicle (V) control for panels *b* and *d* and by a Student's t-test for panel *c*.

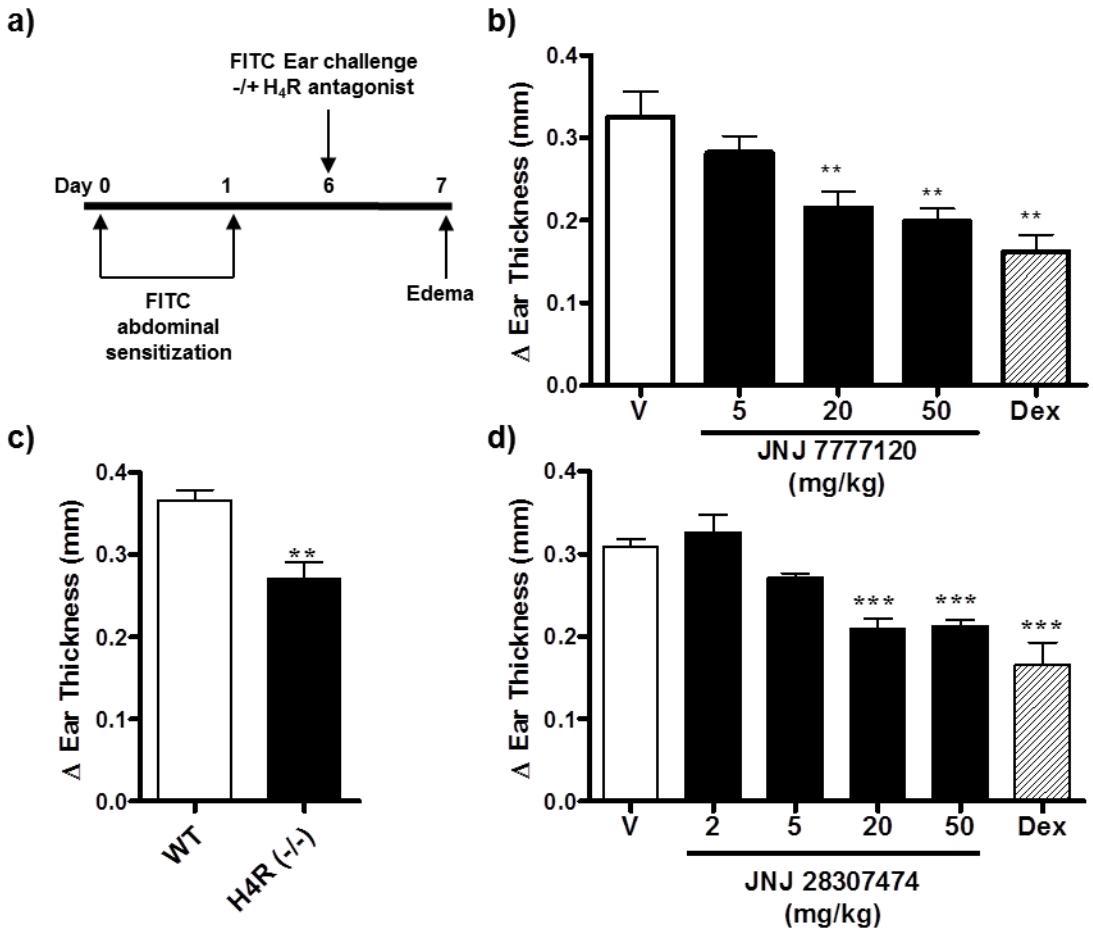

a)
FITC Ear challenge
-/+ H4R antagonist
Day 0
1
6
7
FITC
abdominal
sensitization
Edema
b)
Δ Ear Thickness (mm)
0.4
0.3
0.2
0.1
0.0
**
**
**
V
5
20
50
Dex
JNJ 7777120
(mg/kg)
c)
Δ Ear Thickness (mm)
0.4
0.3
0.2
0.1
0.0
**
WT
H4R (-/-)
d)
Δ Ear Thickness (mm)
0.4
0.3
0.2
0.1
0.0
***
***
***
V
2
5
20
50
Dex
JNJ 28307474
(mg/kg)

**Figure 2. $H_4R$ antagonism reduces inflammation.** Balb/c mice (n = 7-14 mice per group) were sensitized to FITC on days 0 and 1 and then challenged on day six by application of FITC to one ear. On day seven ear specimens were taken for histology from mice that were not exposed to FITC (*a*), mice exposed to FITC and treated with vehicle (*b*) and mice exposed to FITC and treated with 50 mg/kg JNJ 7777120 (*c*). Magnification was 10x for all. *d*, The total severity score was quantitated based on a 0-3 score for inflammation, edema and abscesses. **, $p < 0.01$ by Student's t-test comparing JNJ 7777120 to vehicle control.

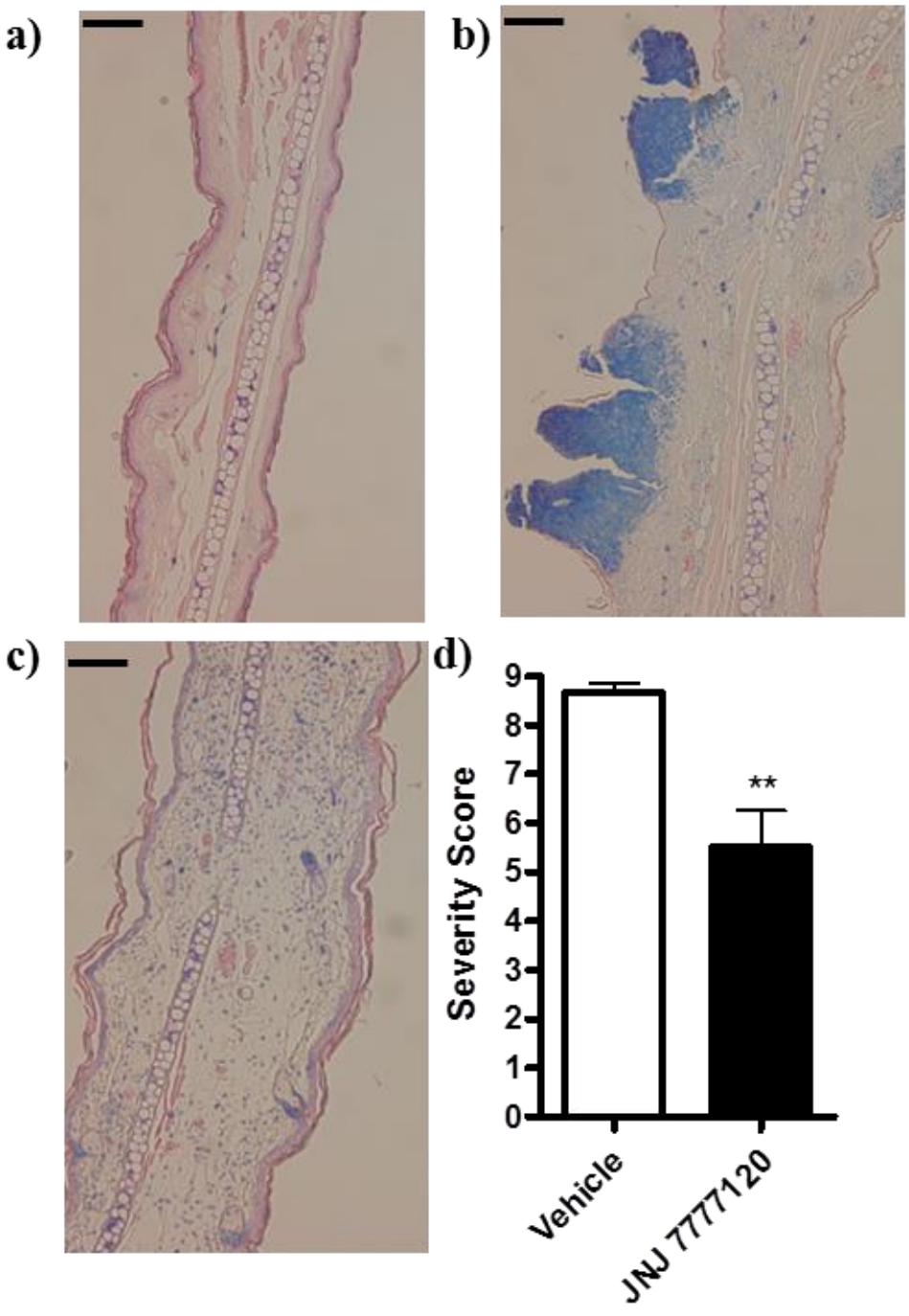

a)
b)
c)
d)
Severity Score
9
8
7
6
5
4
3
2
1
0
**
Vehicle
JNJ 7777120

**Figure 3. $H_4R$ antagonism reduces the infiltration of eosinophils.** Balb/c mice (n = 7-14 mice per group) were sensitized to FITC on days 0 and 1 and then challenged on day six by application of FITC to one ear. On day seven ear specimens were taken for histology and the number of eosinophils quantitated. *a*, Mice exposed to FITC and treated with vehicle and (*b*), mice exposed to FITC and treated with 50 mg/kg JNJ 7777120. Eosinophils are marked with a black arrow and mast cells with a red arrow (60x magnification). *c*, Quantification of eosinophils and mast cells per section. *, $p < 0.05$; ***, $p < 0.001$ by one-way ANOVA with post-hoc Bonferroni's test comparing JNJ 7777120 to vehicle control and α, $p < 0.05$; Ψ, $p < 0.001$ comparing sham to vehicle.

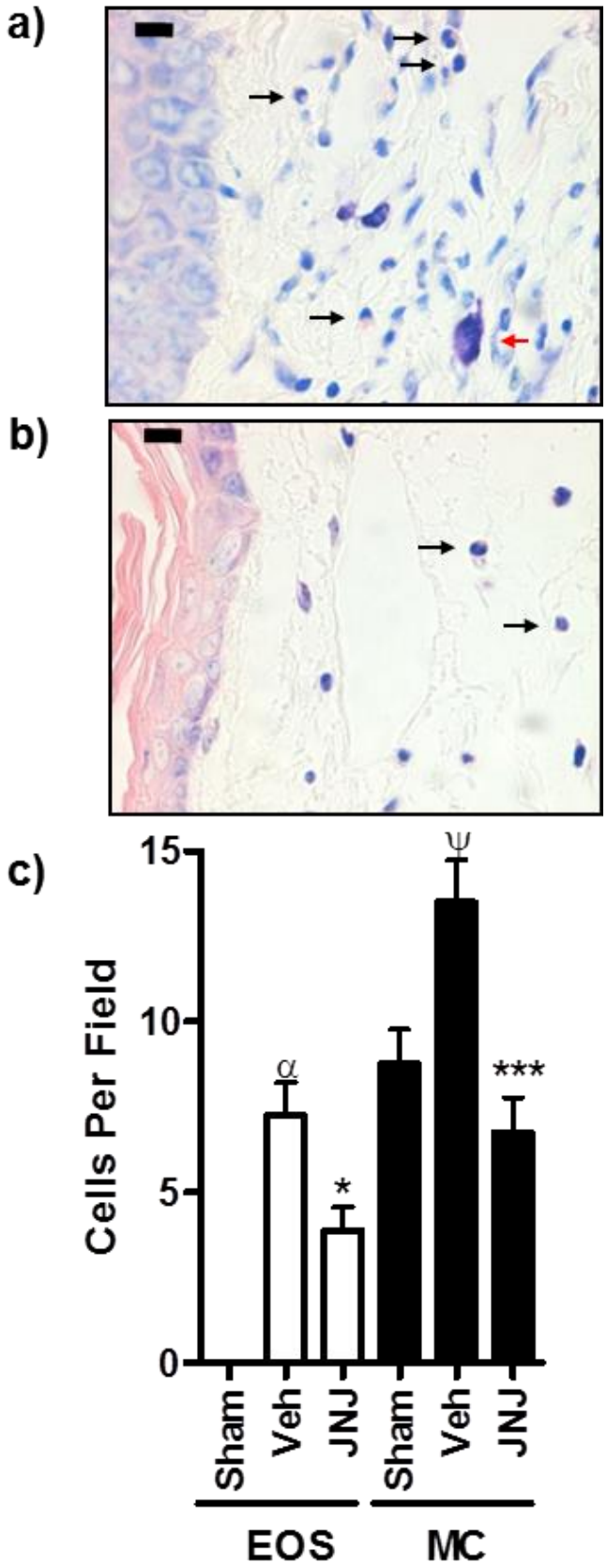

a)
b)
c)
Cells Per Field
15
10
5
0
α
*
Ψ
***
Sham
Veh
JNJ
Sham
Veh
JNJ
EOS
MC

**Figure 4. $H_4R$ antagonism reduces inflammatory cytokines in tissue.** Ear tissues from mice that were sensitized and challenged with FITC were harvested at various times after FITC challenge. The tissues homogenates were analyzed for cytokine and chemokine expression. Mice were either treated with vehicle (white bars) or JNJ 7777120 (50 mg/kg p.o.; black bars). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$ by Student's T-test compared vehicle control at each time point.

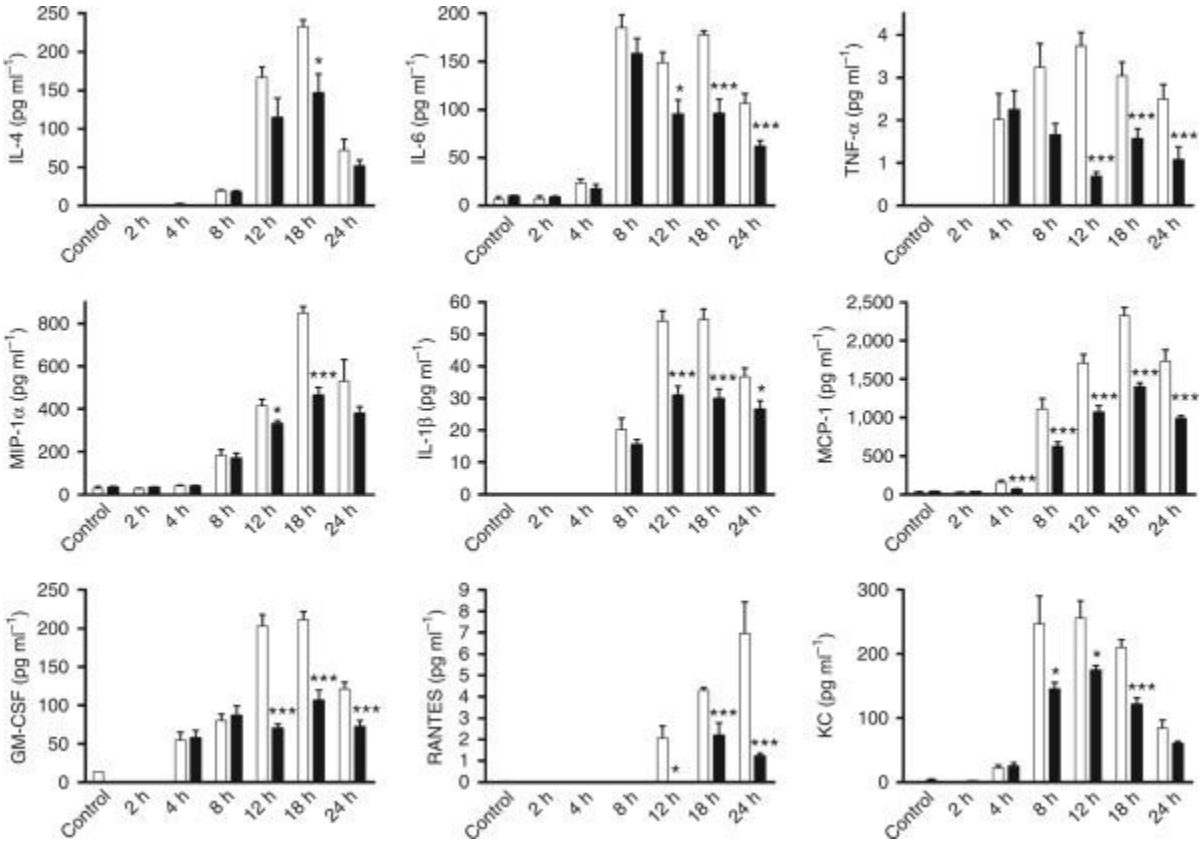

IL-4 (pg ml$^{-1}$)
IL-6 (pg ml$^{-1}$)
TNF-α (pg ml$^{-1}$)
MIP-1α (pg ml$^{-1}$)
IL-1β (pg ml$^{-1}$)
MCP-1 (pg ml$^{-1}$)
GM-CSF (pg ml$^{-1}$)
RANTES (pg ml$^{-1}$)
KC (pg ml$^{-1}$)
Control
2 h
4 h
8 h
12 h
18 h
24 h

**Figure 5. $H_4R$ antagonism reduces Th2 cell responses.** Peripheral lymph nodes were collected 24 h after FITC challenge from mice treated with vehicle (white bars) or JNJ 7777120 (50 mg/kg p.o.; black bars). Lymphocytes were isolated and cultured with either 10 ug/mL FITC or a combination of anti-CD3 and anti-CD28. *a*, Proliferation was measured by $^{3}$H-thymidine incorporation after 96 h. *b,c,d*, IL-4, IL-5 and IL-17 levels were measured after 72 h using ELISA.**, $p < 0.01$ by Student's t-test compared to vehicle control.

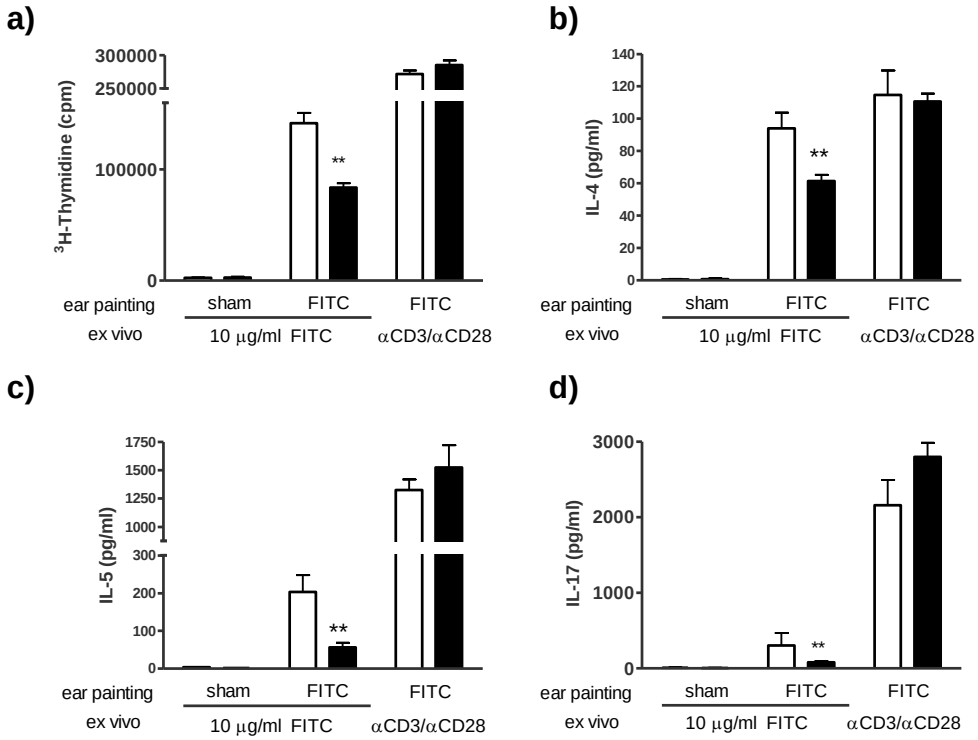

a)
300000
250000
100000
0
$^3$H-Thymidine (cpm)
**
ear painting
sham
FITC
FITC
ex vivo
10 μg/ml FITC
αCD3/αCD28
b)
140
120
100
80
60
40
20
0
IL-4 (pg/ml)
**
ear painting
sham
FITC
FITC
ex vivo
10 μg/ml FITC
αCD3/αCD28
c)
1750
1500
1250
1000
300
200
100
0
IL-5 (pg/ml)
**
ear painting
sham
FITC
FITC
ex vivo
10 μg/ml FITC
αCD3/αCD28
d)
3000
2000
1000
0
IL-17 (pg/ml)
**
ear painting
sham
FITC
FITC
ex vivo
10 μg/ml FITC
αCD3/αCD28

**Figure 6. $H_4R$ antagonism reduces dendritic cell migration *in vivo*.** Peripheral lymph nodes were collected 18 h after FITC challenge from mice treated with vehicle (white bars) or JNJ 7777120 (50 mg/kg p.o.; black bars). Lymphocytes were stained for CD11c and MHC II. FACS analysis was carried out to determine the percentage of $CD11c^+$ $FITC^+$ and MHC $II^+$ $FITC^+$ cells. *a*, representative cytograms. *b*, quantification of the percentage of $CD11c^+$ $FITC^+$ cells. *c*, quantification of the percentage of MHC $II^+$ $FITC^+$ cells. *, $p < 0.05$; **, $p < 0.01$ by Student's t-test compared to vehicle control.

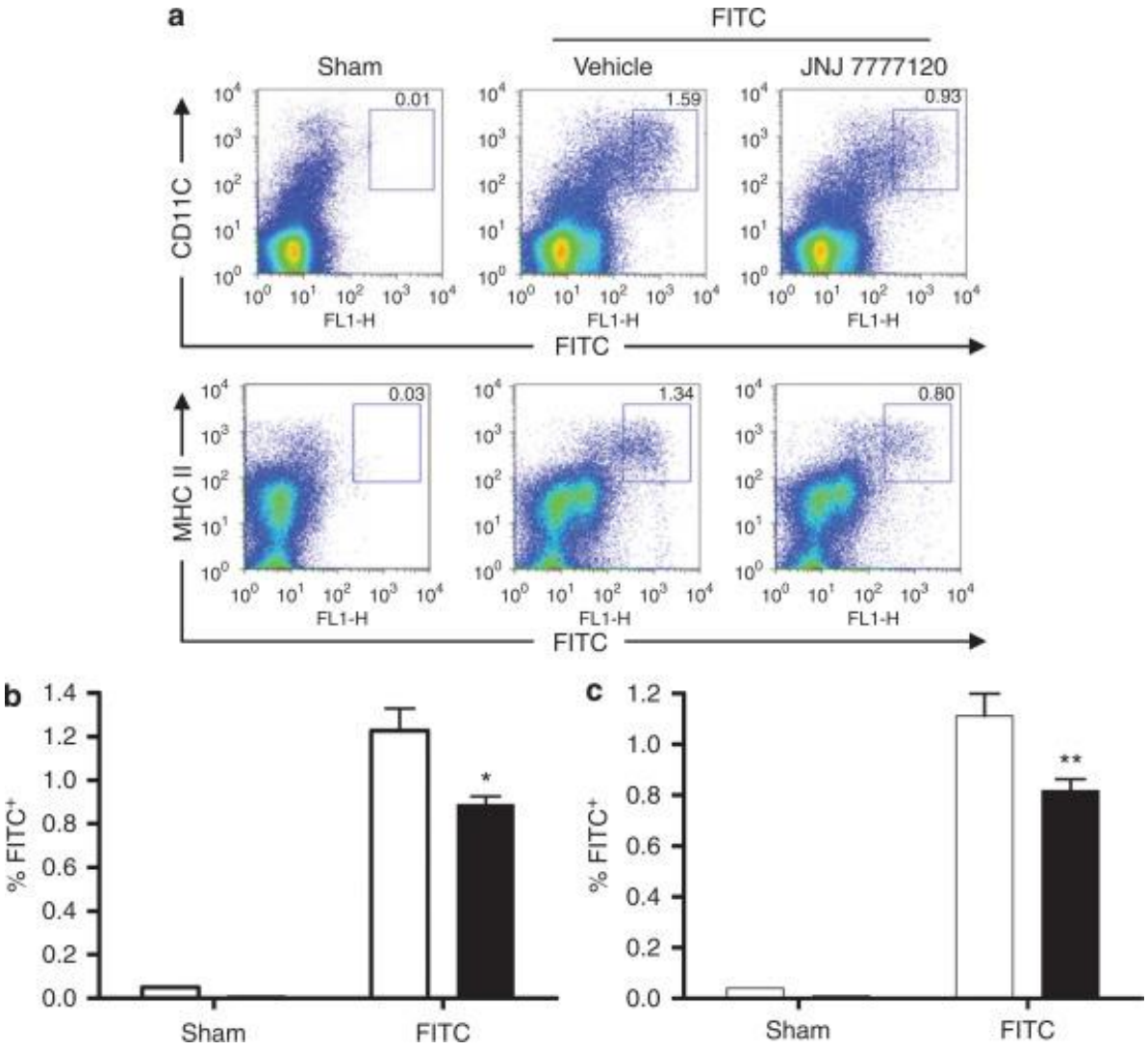

a
FITC
Sham
Vehicle
JNJ 7777120
CD11C
MHC II
FL1-H
FITC
0.01
1.59
0.93
0.03
1.34
0.80
b
% FITC+
Sham
FITC
*
c
% FITC+
Sham
FITC
**

**Figure 7. $H_4R$ antagonism reduces pruritus.** *a*, Balb/c mice (n = 7-14 mice per group) were sensitized to FITC on days 0, 1, 13 and 14 and then challenged on day 20 by application of FITC to one ear. Bouts of scratching were measured for 15 min starting 10 min after FITC application whereas the difference in ear thickness between the challenged and unchallenged ear was measured on day 21. *b*, The $H_4R$ antagonist, JNJ 7777120, given p.o. given 20 min prior to FITC application reduced the pruritus, whereas the $H_1R$ antagonist, fexofenadine (Fex), given at 150 mg/kg p.o. had no effect. *c*, The $H_4R$ antagonist, JNJ 7777120, given p.o. 20 min prior and 4 h after FITC application reduced the swelling induced by FITC challenge in the model given in (*a*), whereas the $H_1R$ antagonist, fexofenadine (Fex), given at 150 mg/kg p.o. had no effect. *d*, *e*, The model given in (*a*) was carried out in mast cell sufficient (WBB6F1 +/+; white bars) and mast cell deficient (WBBF1 $W/W^v$; black bars) mice. The $H_4R$ antagonist, JNJ 7777120, given p.o. 20 min prior and 4 h after FITC application reduced the pruritus (*d*) and edema (*e*) in the mast cell sufficient and deficient mice. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$ by one-way ANOVA with post-hoc Bonferroni's test compared to vehicle (V) control.

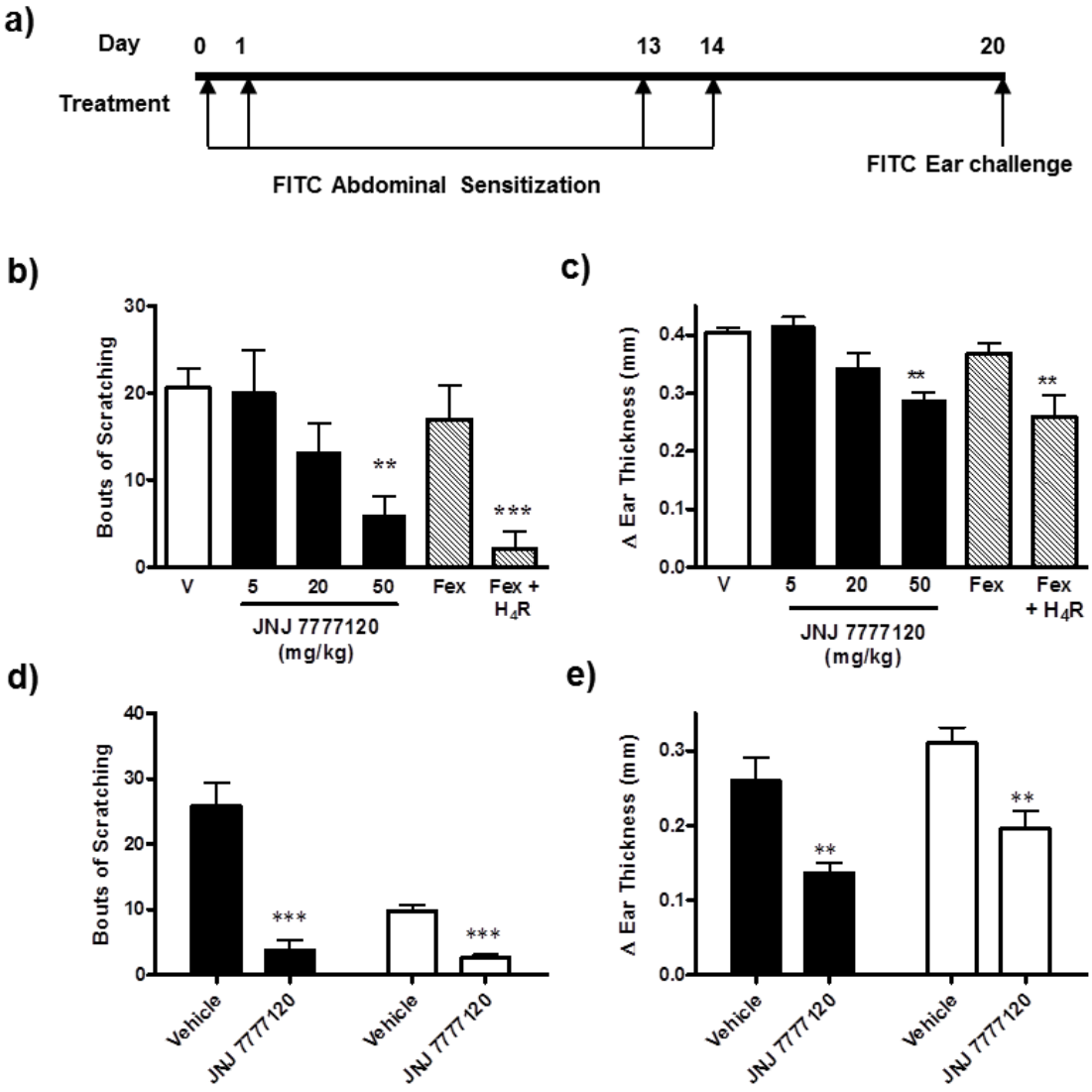
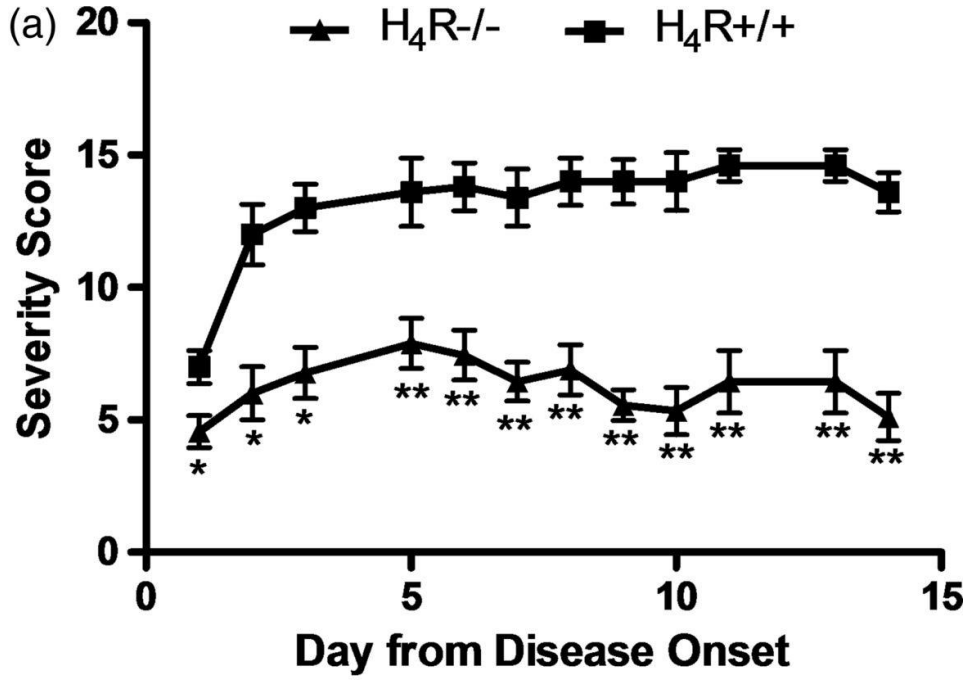

a)
Day
0
1
13
14
20
Treatment
FITC Abdominal Sensitization
FITC Ear challenge
b)
Bouts of Scratching
30
20
10
0
**
***
V
5
20
50
Fex
Fex + H4R
JNJ 7777120 (mg/kg)
c)
Δ Ear Thickness (mm)
0.4
0.3
0.2
0.1
0.0
**
**
V
5
20
50
Fex
Fex + H4R
JNJ 7777120 (mg/kg)
d)
Bouts of Scratching
40
30
20
10
0
***
***
Vehicle
JNJ 7777120
Vehicle
JNJ 7777120
e)
Δ Ear Thickness (mm)
0.3
0.2
0.1
0.0
**
**
Vehicle
JNJ 7777120
Vehicle
JNJ 7777120

**Figure 8**. Comparison of $H_4$R-deficient and wild-type mice in CAIA model (a) $H_4$R-deficient (▲, n = 9) and wild-type (■, n = 5) BALB/c mice were given collagen antibody cocktail and then challenged with LPS ip two days later. Starting the next day, mice were examined visually for the appearance of arthritis in the peripheral joints, and the severity of arthritis was graded on a scale of 0–4 for each paw. The mean and SEM for the sum of the severity scores are given in panel (b). Significant reduction mean disease severity score in $H_4$R-deficient ($H_4$R-/-) compared to wild-type ($H_4$R+/+) can be seen when comparing the area under the curve (AUC, Panel b). Statistical significance was calculated for each time point of the severity score or comparing the AUC using a Mann-Whitney test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.


(a)
$H_4R$-/-
$H_4R$+/+
Severity Score
20
15
10
5
0
0
5
10
15
Day from Disease Onset
*
*
*
**
**
**
**
**
**
**
**

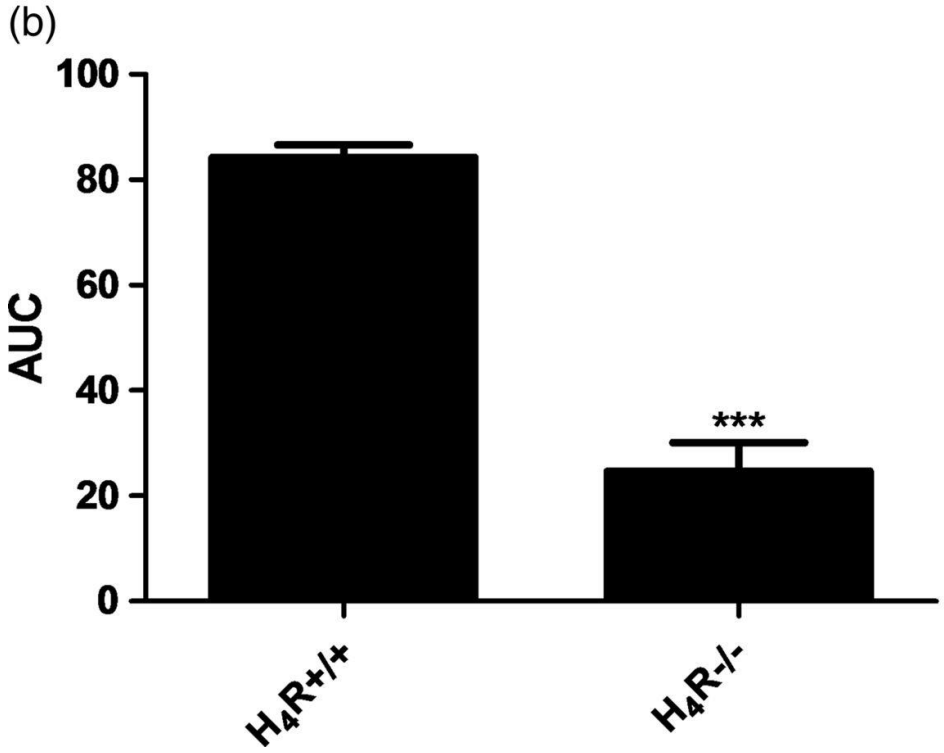
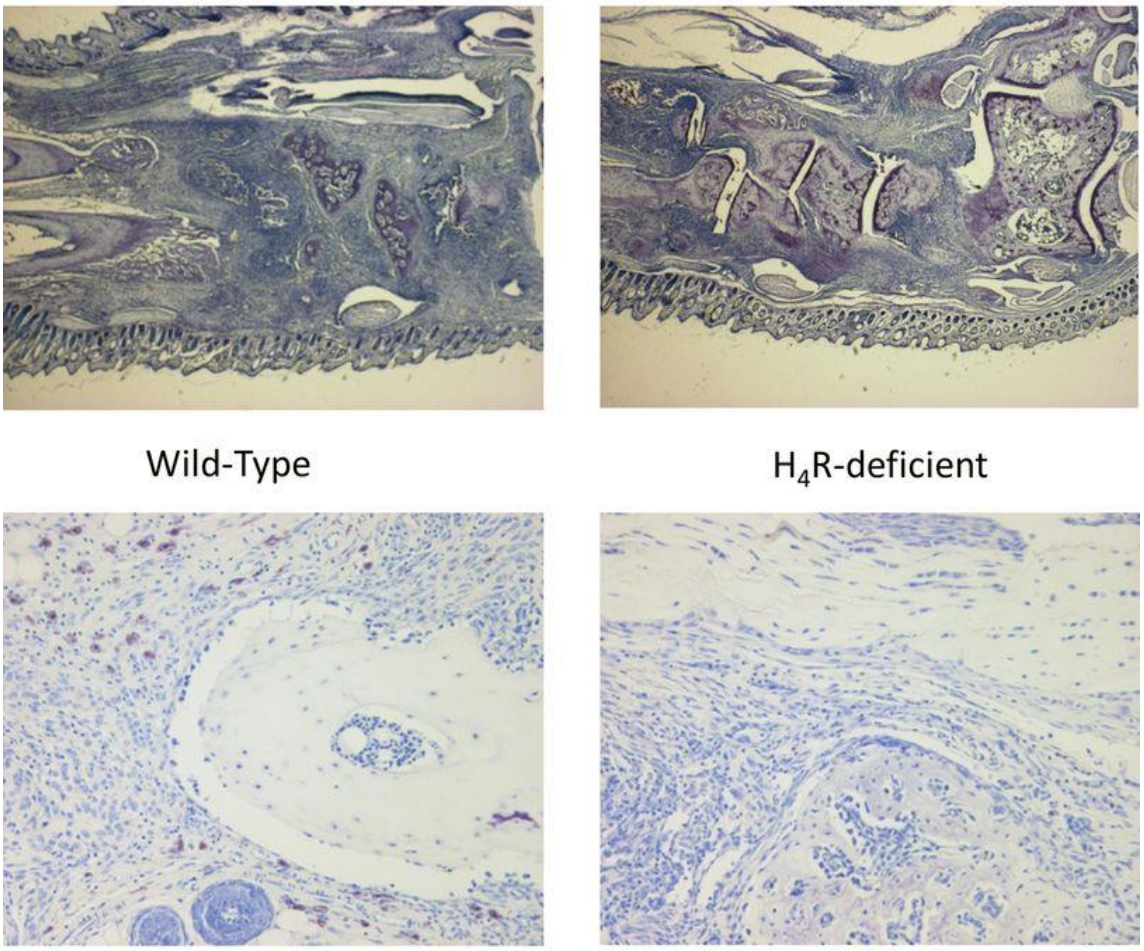

(b)
AUC
100
80
60
40
20
0
***
$H_4R$+/+
$H_4R$-/-

**Figure 9.** Effects on histology in CAIA model (a) Paws were collection for histology and stained with toluidine blue. Representative images are shown for wild-type on the left and $H_4R$-deficient on the right. The top images are a 16x magnification whereas the bottom are 200x. (b) Sections for all animals were scored for inflammation, pannus, cartilage and bone damage and the mean and SEM of these scores are given. Statistical comparison of wild-type (black bars, n = 5) and $H_4R$-deficient mice (white bars, n = 9) was conducted using a Mann-Whitney test. *$p < 0.05$, **$p < 0.01$.

(a)


Wild-Type
H4R-deficient

(b)

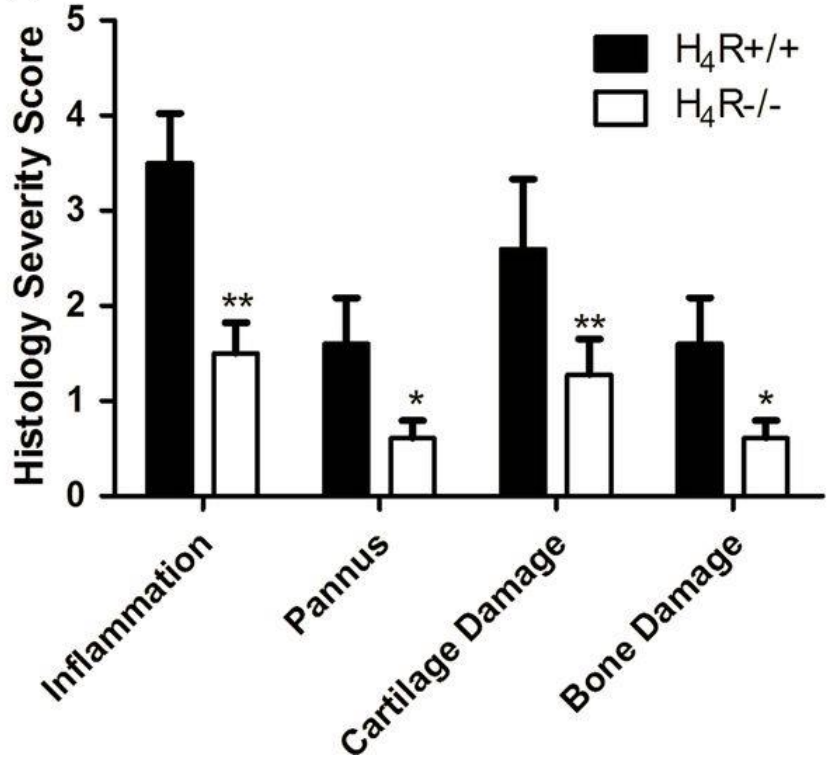

Histology Severity Score
0
1
2
3
4
5
H4R+/+
H4R-/-
**
*
**
*
Inflammation
Pannus
Cartilage Damage
Bone Damage

**Figure 10.** Effects of JNJ 28307474 in CAIA Model. (a) Wild-type BALB/c mice (n = 5 per group) were given collagen antibody cocktail and then challenged with LPS ip two days later. The day after LPS administration mice where treated with vehicle (○), 5 mg/kg (■), 20 mg/kg (▲) or 50 mg/kg (●) JNJ 28307474. All doses were given orally twice a day. The mice were examined visually for the appearance of arthritis in the peripheral joints, and the severity of arthritis was graded on a scale of 0–4 for each paw. The mean and SEM for the sum of the severity scores are given. (b) The area under the curve (AUC) for each time course was calculated and the mean AUC and SEM are given. For both panels statistical significance between each JNJ 28307474 group and vehicle was assessed by a one-way ANOVA with post-hoc Dunnett's test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

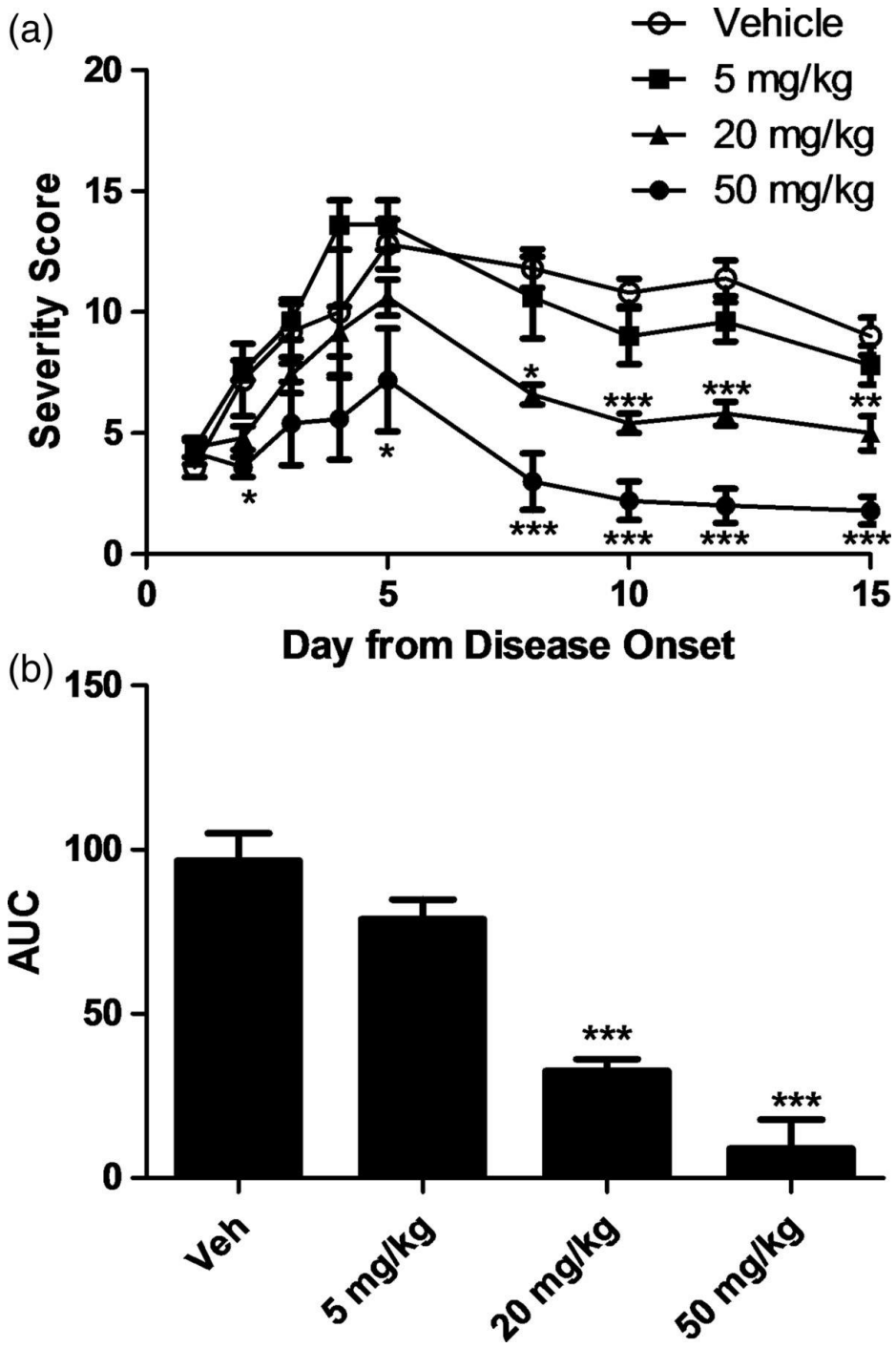
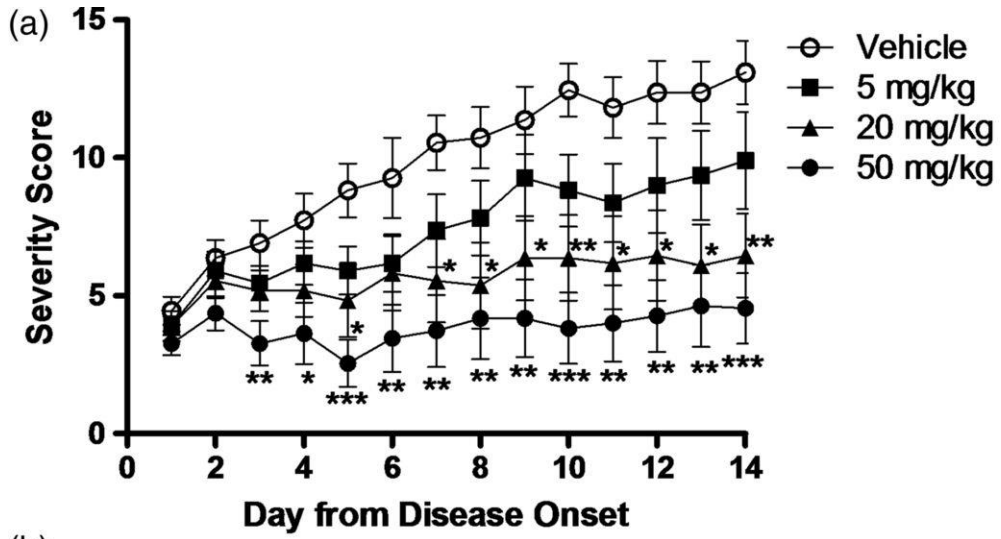
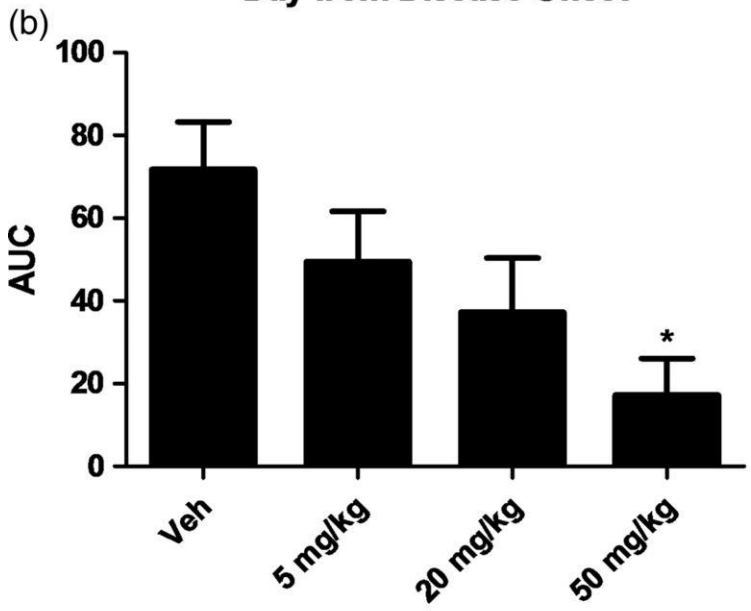

(a)
Vehicle
5 mg/kg
20 mg/kg
50 mg/kg
Severity Score
20
15
10
5
0
0
5
10
15
Day from Disease Onset
*
*
*
***
***
**
***
***
***
***
(b)
AUC
150
100
50
0
***
***
Veh
5 mg/kg
20 mg/kg
50 mg/kg

**Figure 11.** Effects of the $H_4R$ in CIA Model. (a) Wild-type DBA1/J mice (n = 11 per group) were immunized with CFA/collagen and boosted with LPS on day 26. Mice where treated with vehicle (○), 5 mg/kg (■), 20 mg/kg (▲) or 50 mg/kg (●) JNJ 28307474. All doses were given orally twice a day. The mice were examined visually for the appearance of arthritis in the peripheral joints, and the severity of arthritis was graded on a scale of 0–4 for each paw. The mean and SEM for the sum of the severity scores are given. (b) The area under the curve (AUC) for each time course was calculated and the mean AUC and SEM are given. For panels (a) and (b) statistical significance between each JNJ 28307474 group and vehicle was assessed by a one-way ANOVA with post-hoc Dunnett's test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. (c) Wild-type (n = 22) or $H_4R$-deficient (▼, n=12) C57BL/6 mice were immunized twice with CFA/collagen and boosted with LPS on day 28. The mice were examined visually for the appearance of arthritis in the peripheral joints, and the severity of arthritis was graded on a scale of 0–4 for each paw. The mean and SEM for the sum of the severity scores are given. On day 5 wild-type mice were treated orally with vehicle (o, n = 12) or 50 mg/kg twice daily JNJ 28307474 (■, n =10). Statistical significance between $H_4R$-deficient and wild-type mice or JNJ 28307474 and vehicle treated mice was determined using a Mann-Whitney test for each time point. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.


(a)
Severity Score
Day from Disease Onset
Vehicle
5 mg/kg
20 mg/kg
50 mg/kg


(b)
AUC
Veh
5 mg/kg
20 mg/kg
50 mg/kg

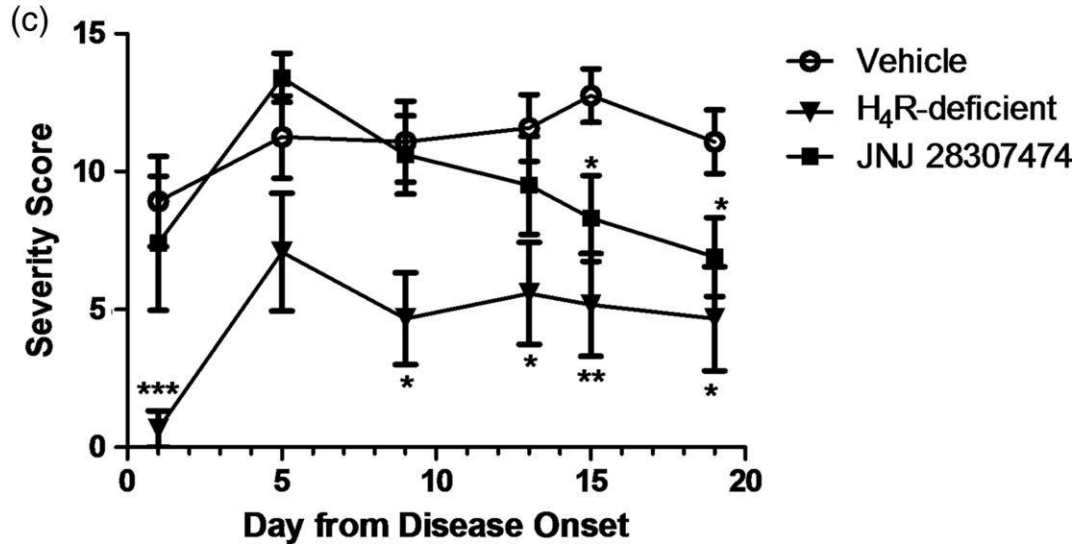
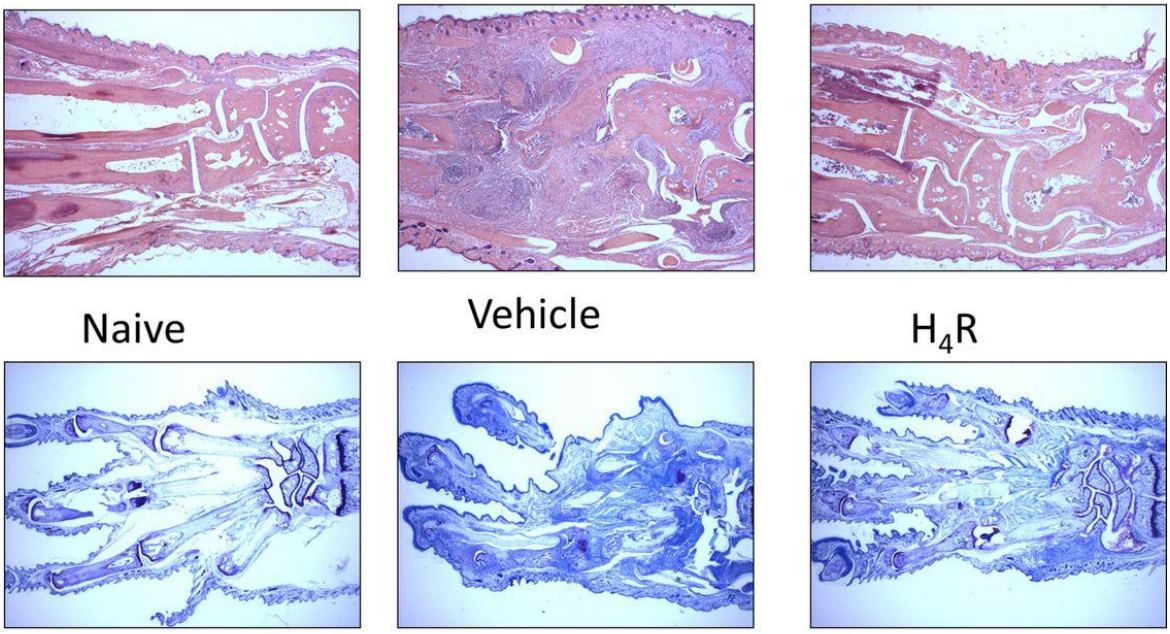

(c)
Severity Score
Day from Disease Onset
Vehicle
H4R-deficient
JNJ 28307474

**Figure 12.** Effects on histology in CIA Model (a) Paws were collection for histology and stained with H&E (top) and toluidine blue (bottom). Representative images are shown for naive (unimmunized) mice (left), immunized and vehicle treated (middle) and immunized and treated with 50 mg/kg JNJ 28307474 orally (right). (b) Sections for all animals were scored for inflammation, pannus, cartilage and bone damage and the mean and SEM of these scores are given. Statistical comparison of treated groups (n = 11 per group) to the vehicle group (n = 11) was assessed by a one-way ANOVA with post-hoc Dunnett's test. *$p < 0.05$.

(a)


Naive
Vehicle
$H_4R$

(b)

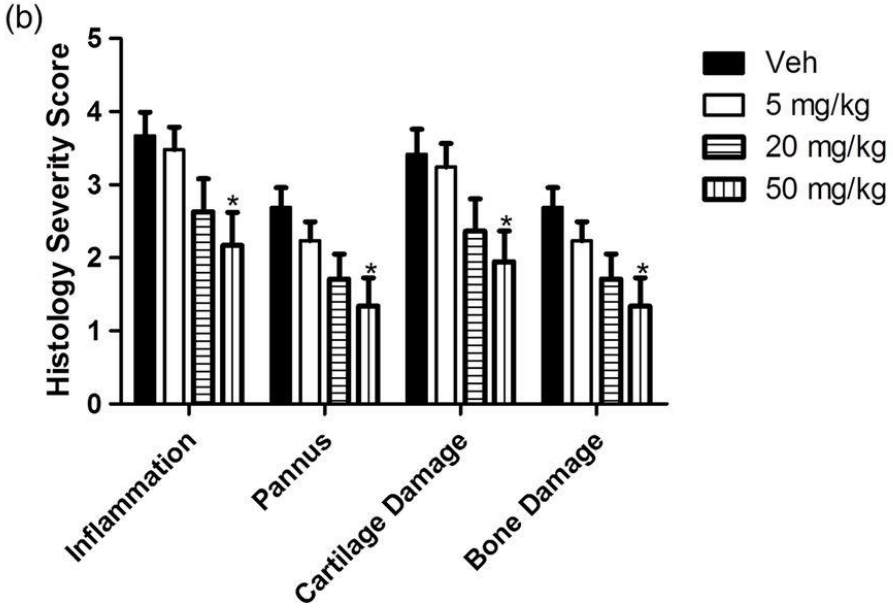
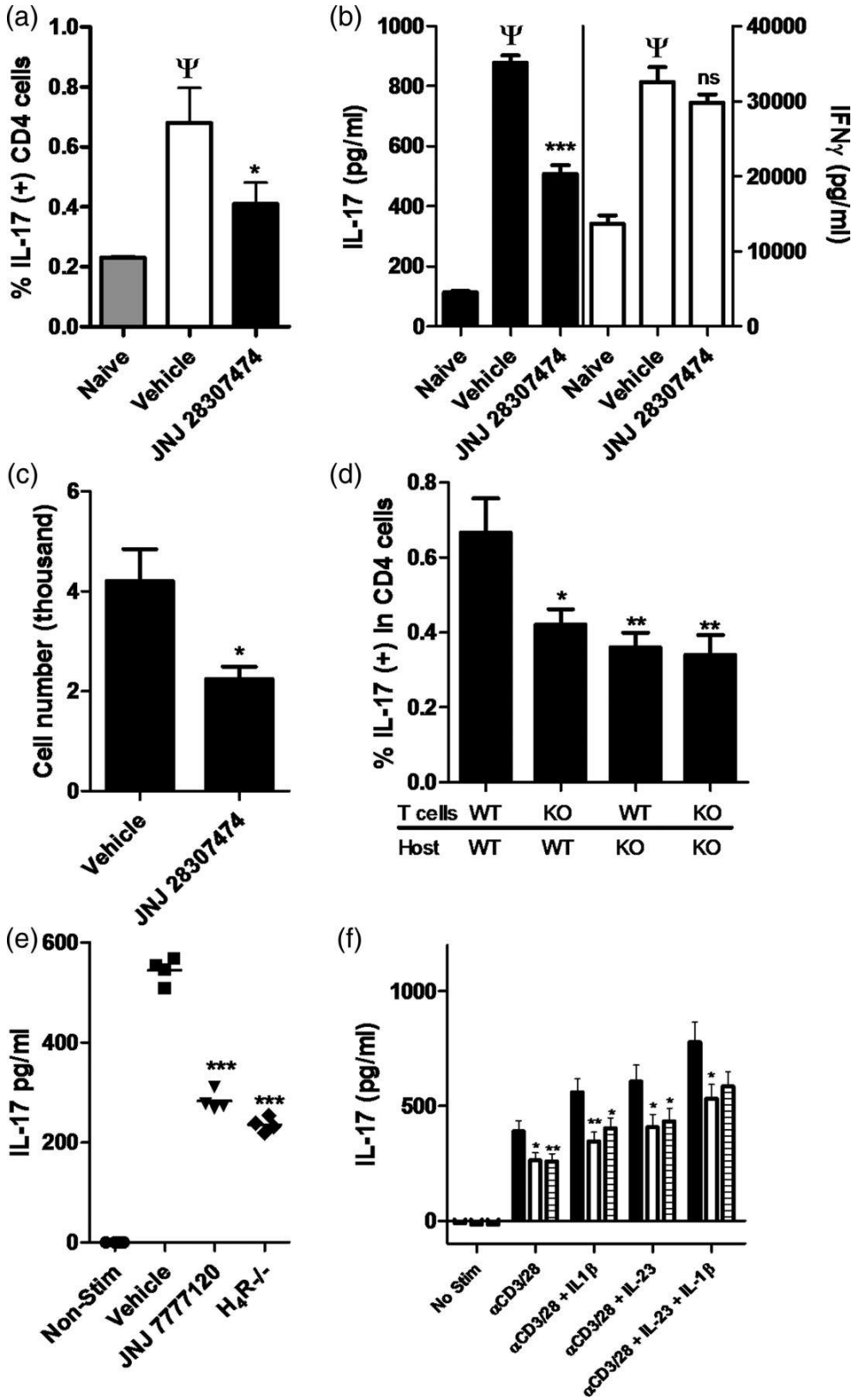

Histology Severity Score
0
1
2
3
4
5
Inflammation
Pannus
Cartilage Damage
Bone Damage
*
Veh
5 mg/kg
20 mg/kg
50 mg/kg

**Figure 13.** Effects of the $H_4R$ on Th17 cells. The percentage of IL-$17^+$ $CD4^+$ cells in the inguinal lymph node (a) and the levels of IL-17 and IFNɤ produced after stimulation with anti-CD3/anti-CD28 (b) were measured in naive (unimmunized) mice, vehicle treatment mice or mice treated orally with 50 mg/kg JNJ 28307474 twice daily. (c) OT-II cells were transferred into wild-type mice one day before immunization with the specific ovalbumin peptide. Starting the day of immunization mice were treated with vehicle (n = 7) or 50 mg/kg twice daily JNJ 28307474 orally (n = 6). Ten days later lymph nodes were harvested and the total number of OT-II $Th17^+$ cells was determined by FACS. (d) Wild-type (WT) or $H_4R$-deficient (KO) OT-II cells were transferred into either wild-type (WT) or $H_4R$-deficient (KO) host mice (n = 6-8 mice per group). One day later the mice were immunized with the specific ovalbumin peptide. Ten days later lymph nodes were harvested and the total number of OT-II $Th17^+$ cells was determined by FACS. (e) Blood from $H_4R$-deficient mice ($H_4R$-/-), wild-type mice treated orally for 20 min with 20 mg/kg JNJ 7777120 or vehicle was stimulated *in vitro* with anti-CD3/CD28 and IL-23 for 18 h. IL-17 measured by ELISA. Non-stimulated (Non-Stim) blood from wild-type mice was used as a control. (f) Human PBMC (n = 5 donors; 2-4 replicates each) were incubated vehicle (dimethylsulfoxide, black bars), 1 µM JNJ 7777120 (open bars) or 1 µM JNJ 28307474 (hatched bars) for 1 h and then various stimuli were added as indicated and further incubation for 48 h. IL-17 was measured by ELISA. For panels (a-c) statistical significance was determined by a Mann-Whitney test. *$p<0.05$, ***$p<0.001$, ns is not significant when comparing JNJ 28307474 treatment to vehicle. Ψ $p<0.01$ comparing vehicle to naïve animals. For panels (d-f) statistical significance between groups was assessed by a one-way ANOVA with post-hoc Dunnett's test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.


(a)
% IL-17 (+) CD4 cells
1.0
0.8
0.6
0.4
0.2
0.0
Ψ
*
Naive
Vehicle
JNJ 28307474
(b)
IL-17 (pg/ml)
1000
800
600
400
200
0
IFNγ (pg/ml)
40000
30000
20000
10000
0
Ψ
***
Ψ
ns
Naive
Vehicle
JNJ 28307474
Naive
Vehicle
JNJ 28307474
(c)
Cell number (thousand)
6
4
2
0
*
Vehicle
JNJ 28307474
(d)
% IL-17 (+) In CD4 cells
0.8
0.6
0.4
0.2
0.0
*
**
**
T cells WT KO WT KO
Host WT WT KO KO
(e)
IL-17 pg/ml
600
400
200
0
***
***
Non-Stim
Vehicle
JNJ 7777120
H4R-/-
(f)
IL-17 (pg/ml)
1000
500
0
No Stim
αCD3/28
αCD3/28 + IL1β
αCD3/28 + IL-23
αCD3/28 + IL-23 + IL-1β