The Histamine H$_4$ Receptor Mediates Inflammation and Pruritus in Th2-Dependent Dermal Inflammation

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The role of histamine H$_4$ receptor (H$_4$R) was investigated in a T-helper type 2 (Th2)-cell-mediated mouse skin inflammation model that mimics several of the features of atopic dermatitis. Treatment with two specific H$_4$R antagonists before challenge with 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$_4$R antagonism reduced lymphocyte proliferation and IL-4, IL-5, and IL-17 levels. One explanation for this finding is that lymph nodes from animals dosed with the H$_4$R antagonist, JNJ 7777120, contained a lower number of FITC-positive dendritic cells. The effect of H$_4$R 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 shown in the model. Therefore, the dual effects of H$_4$R 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$_4$R) 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). The H$_4$R exerts profound effects in regulating immune cell functions, that is, chemotaxis, cytokine, and chemokine expression (Huang and Thurmond, 2008). A number of *in vivo* studies have shown H$_4$R involvement in both innate and adaptive immune responses (Thurmond *et al.*, 2004; Varga *et al.*, 2005; Dunford *et al.*, 2006; Coruzzi *et al.*, 2007). In particular, the H$_4$R has been shown to mediate T helper type 2 (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$_4$R a promising immunomodulatory target for the treatment of allergic, autoimmune, and other inflammatory diseases (Huang and Thurmond, 2008; Thurmond *et al.*, 2008). In addition to its effects in inflammation, the H$_4$R has also been

shown to direct pruritic responses in mice (Bell *et al.*, 2004; Dunford *et al.*, 2007; Rossbach *et al.*, 2009). This dual effect on allergic inflammation and pruritus suggests that the H$_4$R 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; Homey *et al.*, 2006; Bieber, 2008). 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 believed to be driven, at least in the early stages, by Th2 cell responses, because lesions show 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 have a more dominant role in chronic lesions (Akdis *et al.*, 2006; Homey *et al.*, 2006; Bieber, 2008).

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 (Phanuphak *et al.*, 1980; Horsmanheimo *et al.*, 1994; Jarvikallio *et al.*, 1997). Antihistamines that target the histamine H$_1$ receptor (H$_1$R) are frequently used for the treatment of atopic dermatitis-associated pruritus, but their

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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, we 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.

RESULTS

H_4R antagonism inhibits edema in a dermal inflammation model

Dermal inflammation was induced in BALB/c mice by topical exposure to FITC (Figure 1a). Mice were sensitized to FITC by
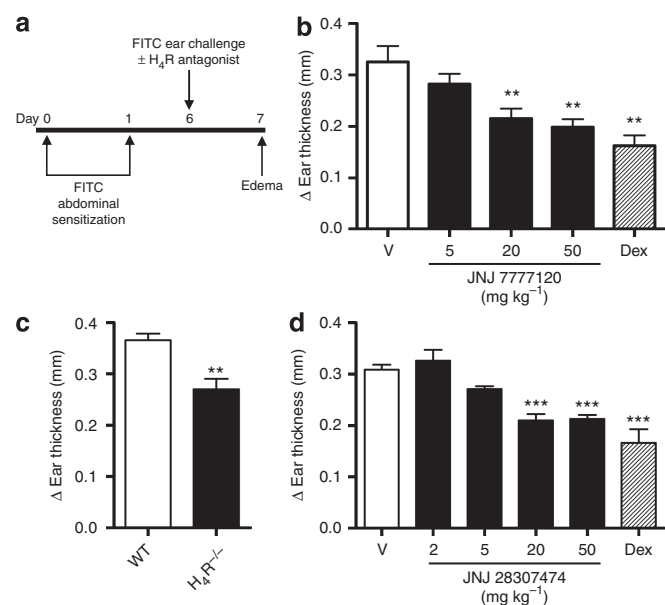
Figure 1. Histamine H_4 receptor (H_4R) antagonism reduces dermal inflammation. (**a**) BALB/c mice ($n = 7$–14 mice per group) were sensitized to FITC on days 0 and 1 and then challenged on day 6 by application of FITC to one ear. On day 7, the difference in ear thickness between the challenged and unchallenged ear was measured with calipers. (**b**) The H_4R antagonist, JNJ 7777120, given p.o. (orally) 20 minutes before and 4 hours 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^{-1}. (**c**) Ear edema was reduced in H_4R-deficient mice ($H_4R^{-/-}$) compared with wild-type (WT) mice. (**d**) Ear edema was also inhibited by a chemically distinct H_4R antagonist, JNJ 28307474, given p.o. 20 minutes before and 4 hours after FITC application. The degree of inhibition by JNJ 28307474 was similar to that of Dex given p.o. at 3 mg kg^{-1}. **$P<0.01$; ***$P<0.001$ by one-way ANOVA using *post hoc* Bonferroni's test compared with vehicle (V) control for panels **b** and **d** and by Student's *t*-test for panel **c**.

painting on the abdomen on 2 consecutive days. After 5 days, FITC was applied to one ear and 24 hours later the ear edema was evaluated as an indication of inflammation. The H_4R-selective antagonist (Thurmond *et al.*, 2004), JNJ 7777120, administered 20 minutes before and 4 hours after FITC application reduced the ear edema in a dose-dependent manner (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^{-1}, p.o. (orally) The role of the H_4R in this model is further supported by the reduction in ear edema in H_4R-deficient mice compared with wild-type mice (Figure 1c). In further support of an H_4R-specific effect, a second H_4R antagonist, JNJ 28307474 (Table 1 and Supplementary Materials), 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). Although this compound does have some cross-reactivity with muscarinic and 5-HT receptors (Supplementary Tables S1 and S2), 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 through the H_4R.

The inflammation in this model was also assessed by histopathological analysis of the ears. At 24 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 and 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 and 3).

In addition, the levels of several cytokines and chemokines from ear homogenates were analyzed. A full time course was carried out and all of the cytokine and chemokines levels peaked at 12–18 hours (Figure 4). FITC application led to

Table 1. *In vitro* K_i 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	2,501
H_1	Mouse	$1,224 \pm 208$
H_1	Guinea pig	3,050
H_2	Human	>1,000

[1]Data given as ± SEM if the assay was run at least two times.
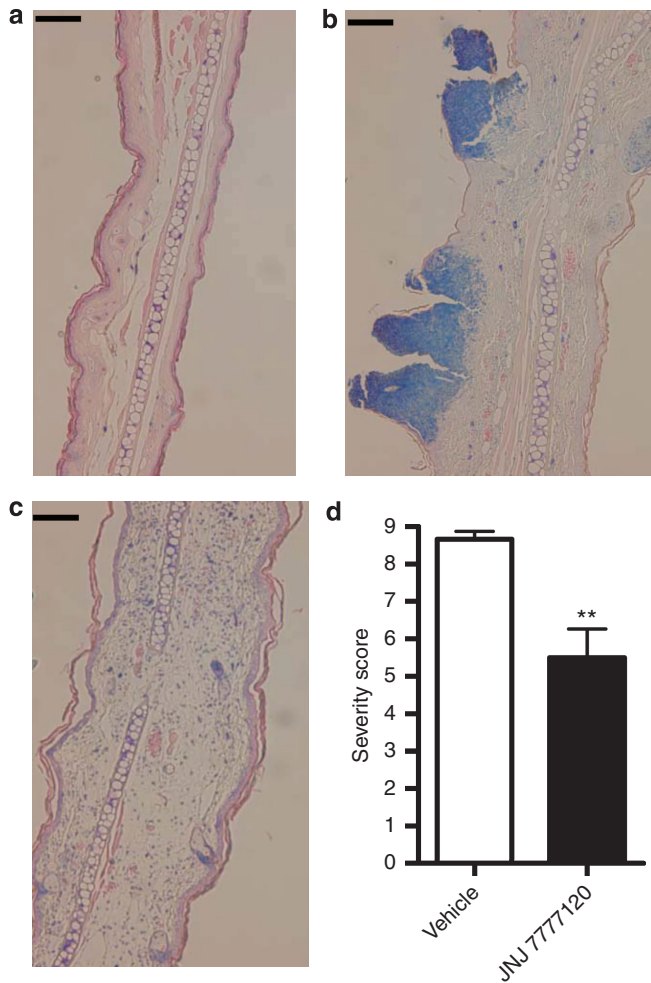
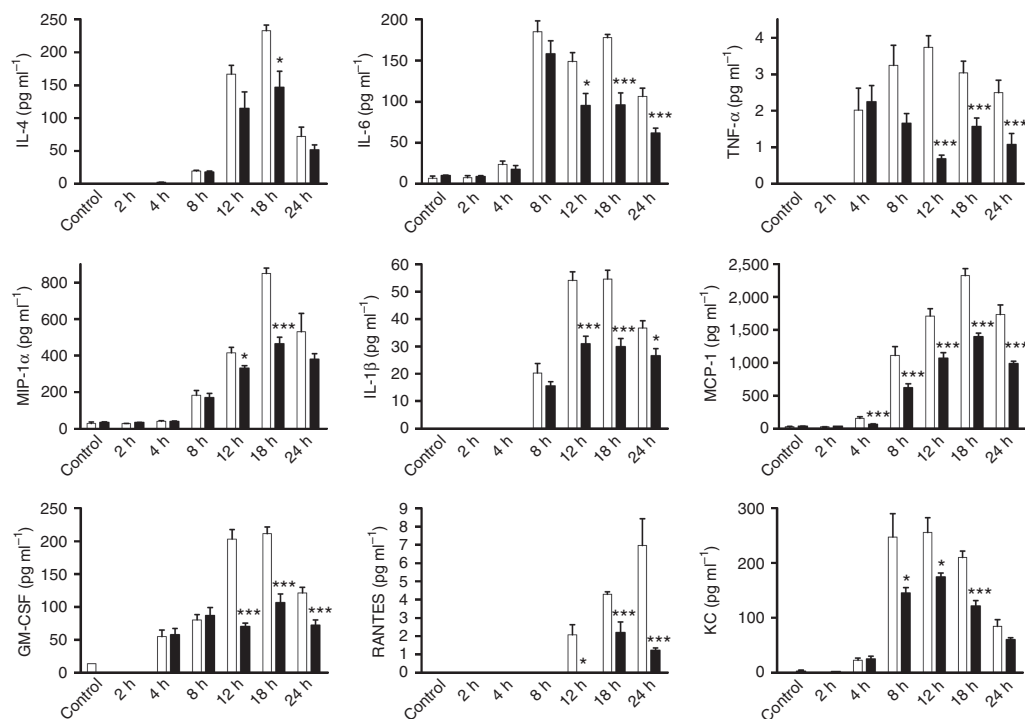**Figure 2. Histamine H[4] receptor (H[4]R) antagonism reduces inflammation.** BALB/c mice ($n = 7$–14 mice per group) were sensitized to FITC on days 0 and 1 and then challenged on day 6 by application of FITC to one ear. On day 7, ear specimens were taken for histology from (**a**) mice that were not exposed to FITC, (**b**) mice exposed to FITC and treated with vehicle, (**c**) and mice exposed to FITC and treated with 50 mg kg^{-1} JNJ 7777120. Bar = 100 μm 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 with vehicle control.

Figure 3. Histamine H[4] receptor (H[4]R) 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 6 by application of FITC to one ear. On day 7, ear specimens were taken for histology, and the number of eosinophils (EOS) was quantitated from (**a**) mice exposed to FITC and treated with vehicle and (**b**) mice exposed to FITC and treated with 50 mg kg^{-1} JNJ 7777120 (JNJ). EOS are marked with a black arrow and mast cells with a red arrow. Bar = 10 μm for all. (**c**) Quantification of EOS and mast cells (MC) per section. *$P < 0.05$ and ***$P < 0.001$ by one-way ANOVA with *post hoc* Bonferroni's test comparing JNJ 7777120 with vehicle control and $^{\psi}P < 0.05$ and $^{\alpha}P < 0.001$ comparing sham with vehicle (Veh).

increases in IL-4 levels, whereas IFN-γ levels were low (30–50 pg ml^{-1}) 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, tumor necrosis factor-α, 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 levels in the ear homogenates were too low to measure. Treatment with the H[4]R antagonist, JNJ 7777120, significantly reduced the levels of MIP-1α, RANTES, IL-4, MCP-1, IL-1β, IL-6, KC, and GM-CSF. Tumor necrosis factor-α was detected at low levels in the FITC-challenged ears and this was significantly reduced by treatment with JNJ 7777120. The inhibition of

Figure 4. **Histamine H₄ receptor (H₄R) 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 treated with either vehicle (white bars) or JNJ 7777120 (50 mg kg⁻¹, p.o.; black bars). *$P<0.05$; and ***$P<0.005$ by Student's t-test compared vehicle control at each time point.

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 H₄R antagonist, JNJ 28307474 (data not shown). These results, along with the effect on ear edema at 24 hours, clearly support a role for the H₄R in Th2-mediated skin inflammation.

Ex vivo antigen restimulation is impaired in H₄R-antagonist-dosed mice

FITC-induced dermatitis in BALB/c mice is suggested to be CD4+ T-cell-dependent and Th2-driven (Dearman and Kimber, 2000), and previous study has shown that the H₄R is involved in Th2 cell activation (Dunford et al., 2006). In order to determine whether the H₄R-mediated inflammation in this model is due to effects on T-cell function, Th2 cytokine levels were measured on 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 with mice whose ears were not challenged with FITC. An equal number of cells were then cultured for 72 hours 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 with anti-CD3/anti-CD28 stimulation, suggesting that the effect was antigen-specific. After antigen restimulation, the production of IL-4, IL-5, and IL-17 was inhibited by treatment in vivo with JNJ 7777120 (Figure 5b–d). This result suggests that H₄R 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 H₄R is involved in Th2-dependent inflammatory responses. In order to better understand the mechanisms involved, we tested the possibility that H₄R has a role in dendritic cell migration, as has been shown in vitro for human monocyte-derived dendritic cells and other cell types (Hofstra et al., 2003; Ling et al., 2004; Gutzmer et al., 2005; Damaj et al., 2007). 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 animals 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 and c). These results suggest that the H₄R is involved in the control of the migration of antigen-carrying dendritic cells to draining lymph nodes, and therefore can affect T-cell priming.
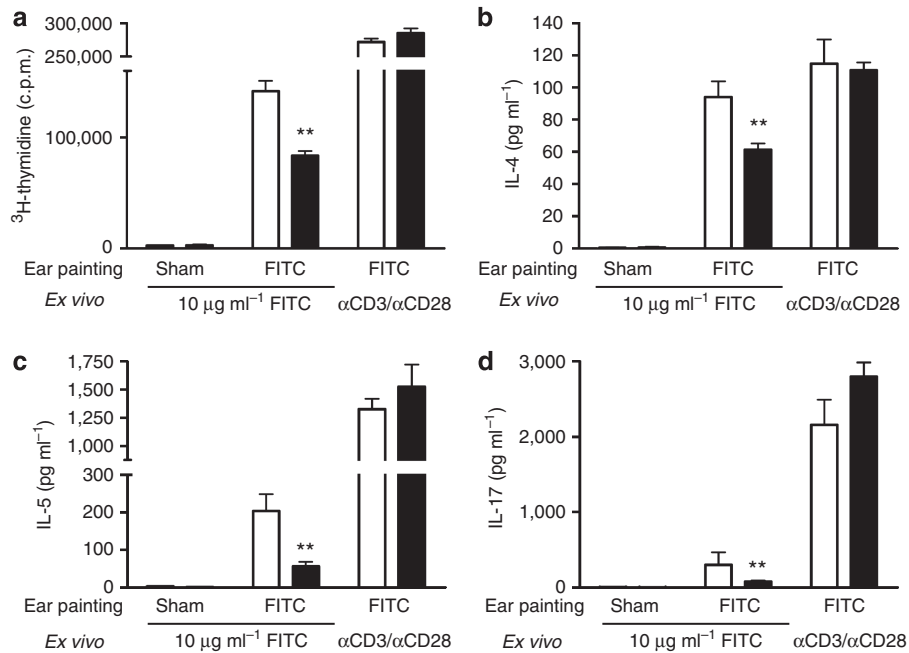
Figure 5. **Histamine H₄ receptor (H₄R) antagonism reduces T helper type 2 (Th2) cell responses.** Peripheral lymph nodes were collected 24 hours after FITC challenge from mice treated with vehicle (white bars) or JNJ 7777120 (50 mg kg^{-1}, p.o.; black bars). Lymphocytes were isolated and cultured with either 10 μg ml^{-1} FITC or a combination of anti-CD3 and anti-CD28. (**a**) Proliferation was measured by ^{3}H-thymidine incorporation after 96 hours. (**b–d**) IL-4, IL-5, and IL-17 levels were measured after 72 hours using ELISA. **$P<0.01$ by Student's t-test compared with vehicle control.

Inflammatory pruritus is inhibited by an H₄R antagonist

Previously, the H₄R has been shown to be involved in mouse models of acute pruritus. Because pruritus is a hallmark feature of many inflammatory skin diseases such as atopic dermatitis, studies were performed to see whether H₄R 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 H₁R antagonist fexofenadine (Fex) given at a dose (150 mg kg^{-1}) 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 hours after the challenge. JNJ 7777120 given 20 minutes before and 4 hours after FITC application on the ear reduced the ear edema in a dose-dependent manner (Figure 7c). The dose response mimicked that seen with pruritus reduction. Again, the effect of the H₁R antagonist Fex in this model was studied and given at a dose of 150 mg kg^{-1} 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 H₄R antagonist, but not the H₁R antagonist, can have both anti-inflammatory and anti-pruritic effects in this model.

To assess whether the effect of the H₄R antagonists was dependent on mast cells, the extended FITC model (Figure 7a) was carried out in mast-cell-sufficient (WBB6F1$^{+/+}$) and mast-cell-deficient (WBBF1 W/W^v) mice. FITC application was able to induce edema and scratching in mast-cell-deficient mice and both could be inhibited by JNJ 7777120 (Figure 7d and e). This indicates that mast cells are not required for the H₄R-mediated responses and that they are not the source of histamine for activating the H₄R. Interestingly, although 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 (Inagaki et al., 2002; Hossen et al., 2003). Recently, it has been recognized that mast cells can have both negative and positive regulatory functions depending on the physiological situation (Galli et al., 2008).

DISCUSSION

Histamine has been implicated in the pathophysiology of atopic dermatitis, but antihistamines that target the H₁R are generally not considered to be effective (Akdis et al., 2006). In this study, we have used a mouse model to test the efficacy of H₄R 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). In this study, we have confirmed the Th2 nature of this model by showing increases in eosinophils, mast cells, and Th2 cytokines in the ear after FITC challenge.
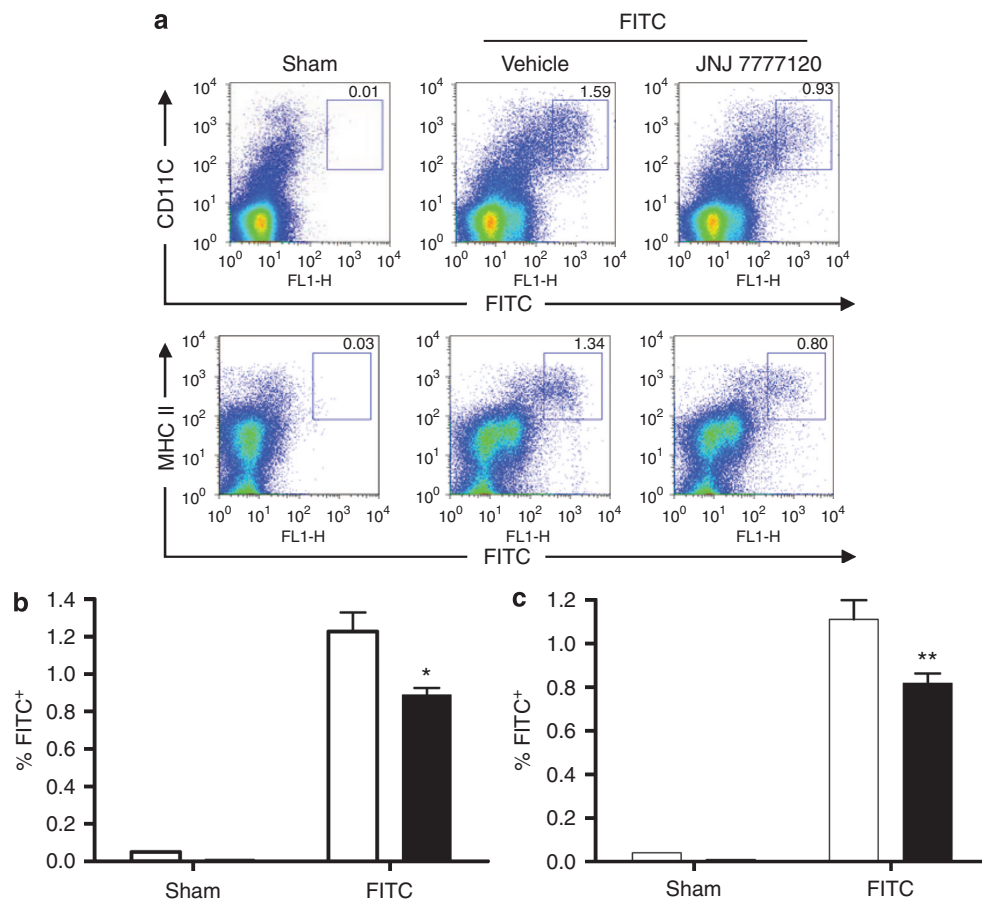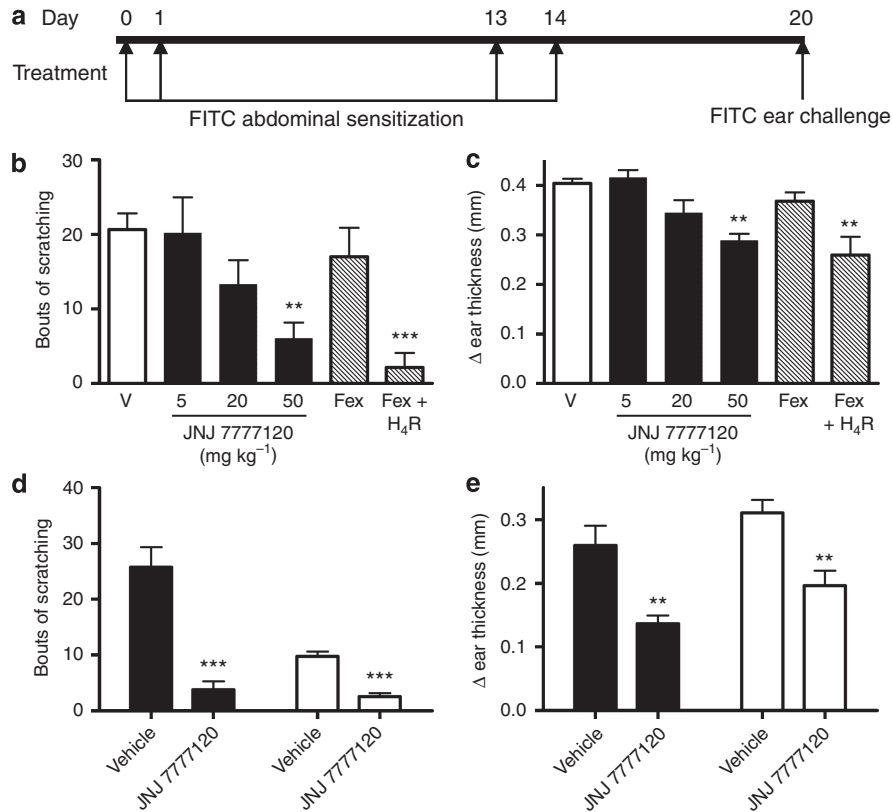
Figure 6. Histamine H$_4$ receptor (H$_4$R) antagonism reduces dendritic cell migration *in vivo*. Peripheral lymph nodes were collected 18 hours after FITC challenge from mice treated with vehicle (white bars) or JNJ 7777120 (50 mg kg^{-1}, p.o.; black bars). Lymphocytes were stained for CD11c and MHC 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. *$P < 0.05$ and **$P < 0.01$ by Student's *t*-test compared with vehicle control.

Previous study has suggested that the H$_4$R modulates allergic lung inflammation mainly through its effects on Th2 cell induction. The data shown here show that this is also true for Th2 responses in the skin. The levels of several proinflammatory cytokines and chemokines including IL-4 were increased in ear tissue on FITC challenge and were inhibited by treatment with H$_4$R antagonists. Although the effects of dexamethasone on cytokine production were not studied, it has recently been shown that it also 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 tumor necrosis factor-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 on exposure to FITC and the levels of both of these could be reduced by treatment with H$_4$R antagonists. The effects on mast cells and eosinophils could be an indirect effect because of changes in cytokine or chemokine levels, or could be a direct effect as the H$_4$R 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$_4$R antagonist (Thurmond *et al.*, 2004).

A direct role for the H$_4$R 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 with that from 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 on FITC stimulation, but not on anti-CD3/anti-CD28 stimulation, was significantly inhibited in lymph nodes taken from animals given JNJ 7777120. In addition, IL-17 was also produced on 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 with regard to the lung, the H$_4$R can modulate Th2 T-cell responses in the skin.

Figure 7. Histamine H₄ receptor (H₄R) 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 minutes starting 10 minutes after FITC application, whereas the difference in ear thickness between the challenged and unchallenged ear was measured on day 21. (**b**) The H₄R antagonist, JNJ 7777120, given p.o. 20 minutes before FITC application reduced the pruritus, whereas the H₁R antagonist fexofenadine (Fex) given at 150 mg kg⁻¹, p.o. had no effect. (**c**) The H₄R antagonist, JNJ 7777120, given p.o. 20 minutes prior to and 4 hours after FITC application reduced the swelling induced by FITC challenge in the model given in panel **a**, whereas the H₁R antagonist Fex given at 150 mg kg⁻¹, p.o. had no effect. (**d** and **e**) The model given in panel **a** was carried out in mast-cell-sufficient (WBB6F1 $^{+/+}$; white bars) and mast-cell-deficient (WBBF1 W/Wᵛ; black bars) mice. The H₄R antagonist, JNJ 7777120, given p.o. 20 minutes prior and 4 hours after FITC application reduced the pruritus (**d**) and edema (**e**) in the mast-cell-sufficient and -deficient mice. **$P < 0.01$; ***$P < 0.005$ by one-way ANOVA with *post hoc* Bonferroni's test compared with vehicle (V) control.

In the previous study, it was shown that H₄R on dendritic cells was necessary for proper stimulation of Th2 cells *in vitro* (Dunford *et al.*, 2007). Therefore, some of the effects of H₄R antagonists on *in vivo* Th2 responses may be directly related to the activation of Th2 cells. In addition to this, it appears that the H₄R 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 H₄R. Recently, it has been shown that the H₄R 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 H₄R 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 H₄R 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 H₄R antagonism. In particular, tumor necrosis factor-α, IL-1β, 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.*, 1997, 1999, 2000, 2003; Smith *et al.*, 1998; Mizumoto *et al.*, 2001; Suto *et al.*, 2006). In addition to the potential indirect effect, histamine acting through the H₄R directly on human monocytes-derived dendritic cells or mouse bone-marrow-derived dendritic cells has been shown to induce chemotaxis *in vitro* (Gutzmer *et al.*, 2005; Damaj *et al.*, 2007; Bäumer *et al.*, 2008). Furthermore, Bäumer *et al.* (2008) have recently shown that histamine can enhance dendritic cell migration from mouse ear explants and that this effect could be blocked by JNJ 7777120. 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$_4$R antagonists on Th2 cytokines appeared to translate into a reduction in ear edema on application of FITC. The inhibitory effect on edema is clearly H$_4$R-mediated as two chemically distinct compounds show equivalent effects and similar effects are seen in H$_4$R-deficient mice. However, this reduction was only partial, indicating that only a portion of the edema is H$_4$R-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 hours (Takeshita *et al.*, 2004). Interestingly, the inhibition seen with either H$_4$R antagonist was equivalent to that seen with dexamethasone.

In addition to the anti-inflammatory effects of H$_4$R 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 the diseased skin of atopic dermatitis patients compared with those in 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$_1$R antihistamines are generally considered to be ineffective in the treatment of atopic dermatitis-associated pruritus (Klein and Clark, 1999; Akdis *et al.*, 2006). Previous study has shown that the H$_4$R 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 be due to a reduction in inflammation or due to a direct effect on sensory neurons, as postulated for the acute pruritus models.

It is of interest that both the edema and pruritic responses to FITC are retained in the W/W^v mice and that the H$_4$R antagonist was still able to block both responses. This suggests that mast cells are not required for either the H$_4$R-mediated edema or pruritic responses, although care should be take with this interpretation as 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 (Tsai *et al.*, 2005; Nigrovic *et al.*, 2008). The mast-cell-independent effects of the H$_4$R on the edema and pruritus in this model are consistent with previous data in a mouse asthma model and other pruritus models (Dunford *et al.*, 2006, 2007). However, the mechanism for the 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$_4$R can mediate the production of IL-31 (Gutzmer *et al.*, 2009) and this cytokine has been linked to pruritus in atopic dermatitis (Dillon *et al.*, 2004; Castellani *et al.*, 2006). In addition, these data suggest that mast cells are not the source of histamine that activates the H$_4$R. Several other cell types in the skin have been shown to have the capacity to produce histamine on stimulation including dendritic cells and keratinocytes (Malaviya *et al.*, 1996; Dunford *et al.*, 2006).

In this study, it is shown that the H$_1$R antagonist Fex 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 Fex, on itch in atopic dermatitis patients (Klein and Clark, 1999). Furthermore, the fact that the H$_1$R antagonist cannot block the FITC-induced edema indicates that histamine itself is probably not driving this response, as this dose of Fex can completely inhibit histamine-induced edema (Dunford *et al.*, 2007). However, these conclusions should be taken with caution because they may depend on the H$_1$R antagonist used. For example, diphenhydramine has been shown to block histamine and antigen-induced itch, which is believed to be due to its central activity (Dunford *et al.*, 2007; Rossbach *et al.*, 2009). It has also been shown that loratadine can inhibit histamine-induced itch, whereas Fex does not; however, neither appear to inhibit compound 48/80-induced scratching (Hossen *et al.*, 2005; Dunford *et al.*, 2007). Whether these differences have to do with the differences in distribution such as central nervous system penetration or perhaps non-H$_1$R-related effects of the compounds is not known.

While this paper was being prepared, 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 study, JNJ 7777120 was able to significantly inhibit the pruritus induced by either hapten. However, the edema formation 24 hours after hapten challenge was not affected. The difference between our finding and those reported in this study regarding the anti-inflammatory properties of H$_4$R 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, which is described as inducing a Th1-dependent effect (Rossbach *et al.*, 2009). In particular, the FITC model used in this study 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$_4$R antagonism as it has been shown that eosinophil chemotaxis can be directly mediated by the H$_4$R (Buckland *et al.*, 2003; Ling *et al.*, 2004) 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$_3$R/H$_4$R antagonist, in reducing edema and eosinophil infiltration in another skin inflammation model (Hirasawa *et al.*, 2009).

These data presented in this study show an effective anti-pruritic and anti-inflammation function of H$_4$R antagonists in a mouse model of Th2-dependent skin inflammation. This effect was superior to that of H$_1$R 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$_4$R antagonists on pruritus, inflammation, and Th2-cell responses

point to their therapeutic potential for the treatment of inflammatory skin disorders such as atopic dermatitis.

MATERIALS AND METHODS

Mice

BALB/c mice were obtained from Charles River Laboratories (Wilmington, MA). WBB6F1$^{+/+}$ and WBBF1 W/W^v mice were purchased from Jackson Laboratory (Bar Harbor, Maine). H₄R-deficient mice were generated as previously described (Hofstra et al., 2003) and crossed on to a BALB/c background for at least 10 generations. Age-matched animals were used in all experiments. Mice were housed in community cages on a 12-hour 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 Institutional Animal Care and Use Committee.

Materials

JNJ 7777120 (5-chloro-1*H*-indol-2-yl)-(4-methyl-piperazin-1-yl)-methanone) and JNJ 28307474 (5-fluoro-4-methyl-2-{5-methyl-2-[4-(1-methyl-piperidin-4-yl)-butoxy]-pyridin-4-yl}-1*H*-benzoimidazole were synthesized as previously described (Jablonowski et al., 2003; Arienti et al., 2005). FITC, dexamethasone, Fex, and dibutylphthalate were obtained from Sigma-Aldrich (St Louis, MO). The selectivity of JNJ 7777120 has been previously described (Thurmond et al., 2004). The selectivity of JNJ 28307474 is given in Table 1 and Supplementary Tables S1 and S2. The binding assays were carried out as previously described (Thurmond et al., 2004).

FITC model

Female mice (6- to 8-week olds) were used. The abdomen of each animal was shaved and sensitized by the application of 100 μl of FITC in dibutylphthalate and acetone on 2 consecutive days. At 5 days after 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 contralateral ear was painted with vehicle (dibutylphthalate/acetone). Finally, the thickness of the ears was measured again 24 hours 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, 15, and 16 and challenged on day 21. The thickness of the ears was measured again 24 hours after FITC application.

Compound administration

All compounds were formulated in 2-hydroxypropyl-β-cyclodextrin for all experiments and were administered p.o. 20 minutes before FITC challenge and 4 hours after challenge.

Histology

For histological examination, ear specimens were fixed in 10% buffered formalin and embedded longitudinally in paraffin by standard methods. Sections of 4 μm thickness 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 <40% of the length of the skin;
- 2: inflammatory cells present between 40 and 80% of the length of the skin; and
- 3: inflammatory cells present along >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 20–40%; and
- 3: increase in relative thickness from normal more than 40%.

Abscesses.

- 0: no abscesses;
- 1: <2;
- 2: <4; and
- 3: ⩾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 six whole sections from each animal. In addition, the number of eosinophils and mast cells were quantitated from eight randomly selected high-power fields from four 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 phosphate-buffered saline (PBS) plus Complete 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, Austin, TX) with a Mouse Cytokine LINCOplex Panel (Millipore, Billerica, MA) as per the 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 (5×10^5 cells per 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^{-1} FITC (diluted from 10 mg ml^{-1} stock in 100% DMSO) for 96 hours. Cell culture supernatants were collected after 3 days, and cells were continued in culture with [^{3}H]-thymidine (1 μCi per well) for another 18 hours 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) with a Bio-Rad Bioplex (Hercules, CA) or Mouse Cytokine LINCOplex Panel (Millipore) as per the 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 per well of 10 μg ml^{-1} anti-mouse CD3 (NA/LE) (BD Pharmingen, San Diego, CA) in PBS, incubated overnight at 4 °C. Before adding cells, the

wells were aspirated and washed twice with PBS. After the addition of cells (5×10^5 cells per well), anti-mouse CD28 (NA/LE) (BD Pharmingen) was added to a final concentration of $2 \, \mu g \, ml^{-1}$ and incubated for 72 hours 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 2 consecutive days. At 5 days after the sensitization, animals were dosed with compounds, and 30 minutes 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). At 24 hours after the application of FITC, auricular lymph nodes were removed, and digested with 2.5 ml digestion buffer (RPMI 1640 containing 50 μl of DNase I (Sigma-Aldrich) and Liberase III (Roche Applied Science, Indianapolis, IN)) for 20 minutes at 37 °C. Fresh digestion buffer (2.5 ml) was added and incubated for another 15 minutes at 37 °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 1×10^7 cells per ml and $1–2 \times 10^6$ cells were used for each staining. Cells were incubated with 1:50 FcR blocker (BD Pharmingen, San Diego, CA) on ice for 15 minutes, stained with 1:50 PE-anti-CD11c or PE-anti-I-A^d (BD Pharmingen) for 45 minutes on ice. Cells were washed once with 3 ml FACS buffer, resuspended, and collected on FACSCalibur (BD Biosciences Immunocytometry Systems, San Jose, CA). Before analysis, $1 \, \mu g \, ml^{-1}$ propidium iodide was added to stain for dead cells.

Pruritus assessment

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

CONFLICT OF INTEREST

All authors are employees of Johnson & Johnson. The authors state no conflict of interest.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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