

IL-21 Reduces Immediate Hypersensitivity Reactions in Mouse Skin by Suppressing Mast Cell Activation or IgE Production

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IL-21 regulates activation, proliferation, and differentiation of various immune cells. We have previously shown that exogenous IL-21 administration reduces allergic reactions in mouse models of anaphylaxis and allergic rhinitis. However, the effects of IL-21 in allergic cutaneous reactions remain unclear. In this study, we examined the effects of IL-21 in a mouse model of the IgE-mediated cutaneous immediate hypersensitivity reaction (IHR). We also investigated the mechanism of IL-21-induced regulation of allergic cutaneous reactions. Mice were sensitized by intraperitoneal ovalbumin (OVA) injection and challenged by injecting OVA intradermally into the ears, with intraperitoneal administration of recombinant murine (rm)IL-21 during the sensitization period or after completion of sensitization. After challenge, IL-21-untreated allergic mice developed biphasic responses characterized by early-phase and late-phase reactions. The biphasic reactions were significantly reduced by rmIL-21 treatment during sensitization or after completion of sensitization. Administration of rmIL-21 during sensitization reduced the cutaneous IHR by suppressing allergen-specific IgE production. In contrast, administration of rmIL-21 after completion of sensitization did not decrease serum levels of allergen-specific IgE, but significantly suppressed mast cell degranulation in skin. These results suggest that the regulatory effects of IL-21 on the cutaneous IHR involve suppression of allergen-specific IgE production or mast cell degranulation.

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INTRODUCTION

The immediate hypersensitivity reaction (IHR) is involved in the pathogenesis of many cutaneous allergic diseases such as urticaria, latex allergy, and atopic dermatitis. Treatment of these allergic diseases is usually performed with corticosteroids and antihistamines, but both of these drug classes have limitations. Corticosteroids are powerful but relatively nonspecific anti-inflammatory agents that sometimes cause side effects such as increased susceptibility to infections. Antihistamines antagonize several chemical mediators, but severe symptoms are often resistant to these drugs. Therefore, new therapeutic strategies for the allergic disorders are required.

When patients with an IHR are challenged with an allergen against which they are sensitized, they often develop a biphasic immune reaction. Following allergen stimulation, B cells differentiate into Ig-producing cells and secrete allergen-specific IgE. Subsequent exposure to the allergen induces activation of mast cells via crosslinking of high-affinity IgE receptors bound to IgE with the allergen (Galli *et al.*, 2005). The activated mast cells release proinflammatory vasoactive amines (e.g., histamine), arachidonic acid derivatives (e.g., leukotriene (LT)₄), cytokines (e.g., tumor necrosis factor- α (TNF- α)), and chemokines (e.g., regulated on activation, normal T-cell expressed, and presumably secreted (RANTES)) that produce a biphasic allergic reaction. Thus, IgE production or mast cell activation is a key step in development of the IHR, and suppression of these phenomena may provide effective treatment of allergic diseases.

IL-21 is a 4-helix bundle type I cytokine that is produced by activated CD4⁺ T cells (Parrish-Novak *et al.*, 2000) and natural killer T cells (Coquet *et al.*, 2007), and shares the common receptor γ -chain with IL-2, IL-4, IL-7, IL-9, and IL-15 (Parrish-Novak *et al.*, 2000). IL-21 has a wide variety of functions, including regulation of activation, proliferation, and differentiation of T cells, B cells, natural killer cells, and dendritic cells (Brandt *et al.*, 2007; Spolski and Leonard, 2008; Konforte *et al.*, 2009). Interestingly, both the positive and the negative regulatory effects of IL-21 on allergic reactions have been reported. For example, administration of

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Abbreviations: BMMC, bone marrow-derived mast cell; IHR, immediate hypersensitivity reaction; LT, leukotriene; MPO, myeloperoxidase; OVA, ovalbumin; RANTES, regulated on activation, normal T-cell expressed, and presumably secreted; rm, recombinant murine; TNF, tumor necrosis factor

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IL-21 during sensitization decreased eosinophil recruitment into inflamed sites by inhibiting allergen-specific IgE production in a mouse model of allergic airway inflammation (Suto *et al.*, 2002), whereas in IL-21 receptor-deficient mice, allergen-induced eosinophil infiltration was reduced in the inflamed airway (Fröhlich *et al.*, 2007). Studies from our laboratory have revealed that administration of IL-21 reduces allergic reactions by suppressing production of allergen-specific IgE or chemokines in mouse models of anaphylaxis (Kishida *et al.*, 2007) and allergic rhinitis (Hiromura *et al.*, 2007). These findings suggest that exogenous IL-21 suppresses allergen-triggered immune responses by inhibiting allergen-specific IgE production, whereas the absence of IL-21 receptor signaling reduces allergen-induced inflammation.

The above observations prompted us to investigate the potential suppressive effects of exogenous IL-21 on allergic cutaneous inflammation. In this study, we investigated the effects of IL-21 on the IHR in mouse skin during the sensitization period and after completion of sensitization. In addition, we evaluated the effects of IL-21 on allergen-specific IgE production and mast cell degranulation as possible mechanisms underlying IL-21-induced regulatory effects in the cutaneous IHR.

RESULTS

IL-21 administration during sensitization reduces biphasic allergic cutaneous reactions

Mice were sensitized intraperitoneally once with ovalbumin (OVA) and given recombinant murine (rm)IL-21 or vehicle twice a week for 2 weeks during the sensitization period, as shown in Figure 1a. The sensitized mice were then challenged with OVA and the time course of the ear swelling

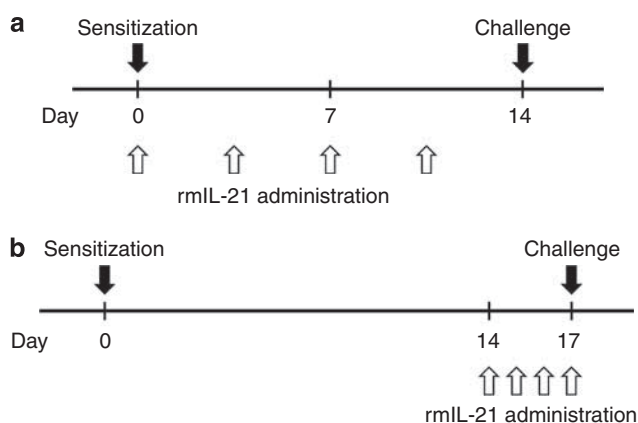


Figure 1. Schemes showing the timing of sensitization, challenge, and recombinant murine (rm)IL-21 administration. (a) Mice were sensitized by intraperitoneal injection of ovalbumin (OVA) and given rmIL-21 or vehicle intraperitoneally twice a week for 2 weeks during the sensitization period. Reactions to OVA were elicited by intracutaneous injection of OVA in the ear on day 14. (b) In separate experiments, mice were sensitized by intraperitoneal injection of OVA and given rmIL-21 or vehicle intraperitoneally once a day on days 14–17. Reactions to OVA were elicited by intracutaneous injection of OVA in the ear at 2 hours after the final rmIL-21 administration on day 17.

response was assessed by measuring ear thickness and weight as indices of edema and cell infiltration. The increase in ear thickness was significant at 1 and 24 hours after allergen challenge in control (IL-21-untreated allergic) mice. Administration of rmIL-21 during sensitization significantly decreased the biphasic ear swelling response (Figure 2a). In the controls, ear weight showed a large increase at 1 hour after challenge and then decreased gradually. Administration of rmIL-21 reduced the increase of ear weight at 1 and 24 hours after challenge (Figure 2b).

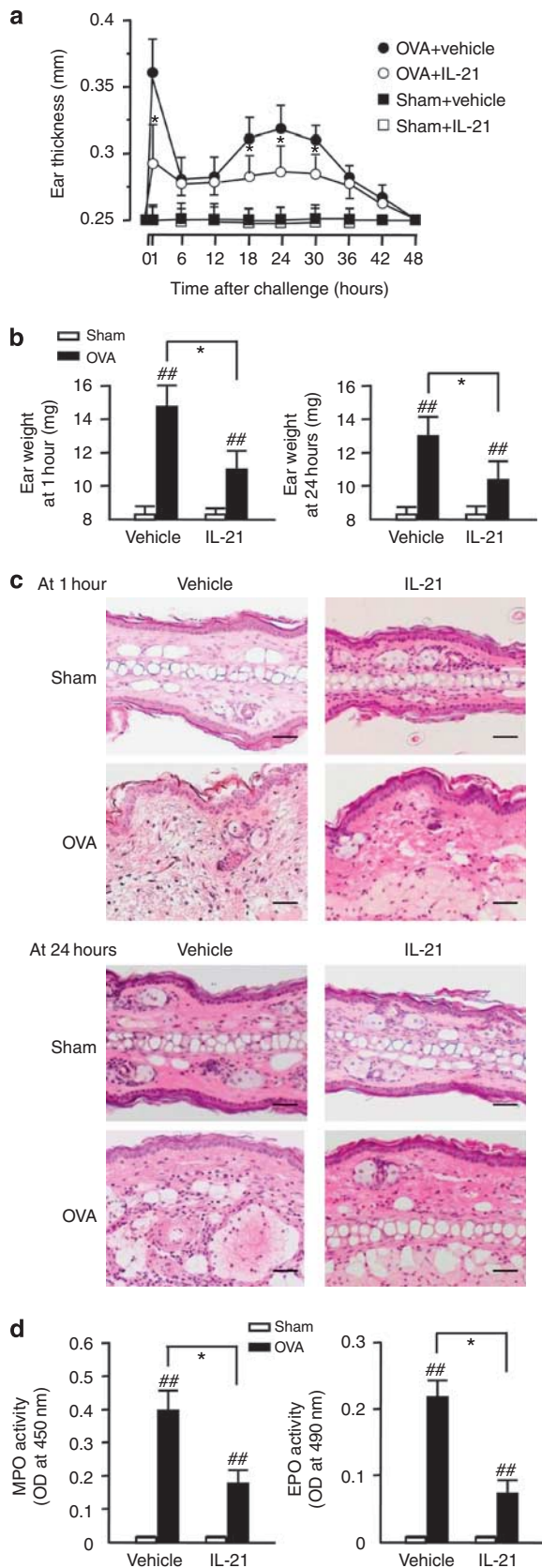
Histological examination of the ear skin revealed marked edema in the dermis at 1 hour after challenge in control mice, but only slight edema in IL-21-treated allergic mice (Figure 2c). At 24 hours after challenge, leukocyte infiltration in the dermis was also greatly decreased in IL-21-treated allergic mice compared with controls (Figure 2c). To quantify leukocyte infiltration into skin in the late phase, myeloperoxidase (MPO) and eosinophil peroxidase activities in the ear skin were determined. In IL-21-treated allergic mice, these activities were significantly lower than those in controls at 24 hours after challenge (Figure 2d). These results show that rmIL-21 administration during the sensitization period significantly reduces biphasic allergic cutaneous reactions.

IL-21 administration after completion of sensitization also reduces biphasic allergic cutaneous reactions

Allergic patients are usually sensitized against one or more allergens, as shown by the presence of specific IgE antibodies to food and environmental allergens. Therefore, we examined the effect of exogenous IL-21 on the IHR in skin after completion of sensitization. Mice were sensitized intraperitoneally once with OVA, and completion of sensitization was confirmed by measuring serum IgE levels 2 weeks later (Supplementary Table S1 online). Mice were given rmIL-21 or vehicle intraperitoneally for 4 consecutive days after completion of sensitization, as shown in Figure 1b. Interestingly, rmIL-21 administration after completion of sensitization also significantly suppressed the biphasic ear swelling responses (Figure 3a). In addition, rmIL-21 greatly reduced the increase in ear weight at 1 and 24 hours after challenge (Figure 3b).

Histological findings in ear skin following rmIL-21 administration showed only slight edema in the dermis in the early phase, compared with control mice (Figure 3c). In the late phase, leukocyte infiltration in rmIL-21-treated allergic mice was significantly lower than that in control mice (Figure 3c). MPO and eosinophil peroxidase activities were also significantly reduced in rmIL-21-treated allergic mice compared with controls (Figure 3d). These results indicate that rmIL-21 administration after completion of sensitization greatly suppressed biphasic allergic cutaneous reactions.

To investigate the role of endogenous IL-21 in skin, we examined expression of IL-21 in inflamed skin in the IHR and the effects of neutralizing endogenous IL-21 in skin on the IHR. Immunohistological analysis revealed no expression of IL-21 in inflamed skin at 1 hour after challenge. In contrast, IL-21 was weakly expressed in the dermis in the IHR at 24 hours (Supplementary Figure S1-a online). To investigate



the effects of anti-IL-21-neutralizing antibody treatment on the IHR, sensitized mice were challenged by injecting intradermally with OVA plus anti-IL-21-neutralizing antibody or isotype-matched control IgG on day 14. Anti-IL-21 treatment resulted in no significant change in ear swelling responses and histological findings compared with controls (Supplementary Figure S1-b online).

Allergen-specific IgE production is suppressed by IL-21 administration during sensitization but not after completion of sensitization

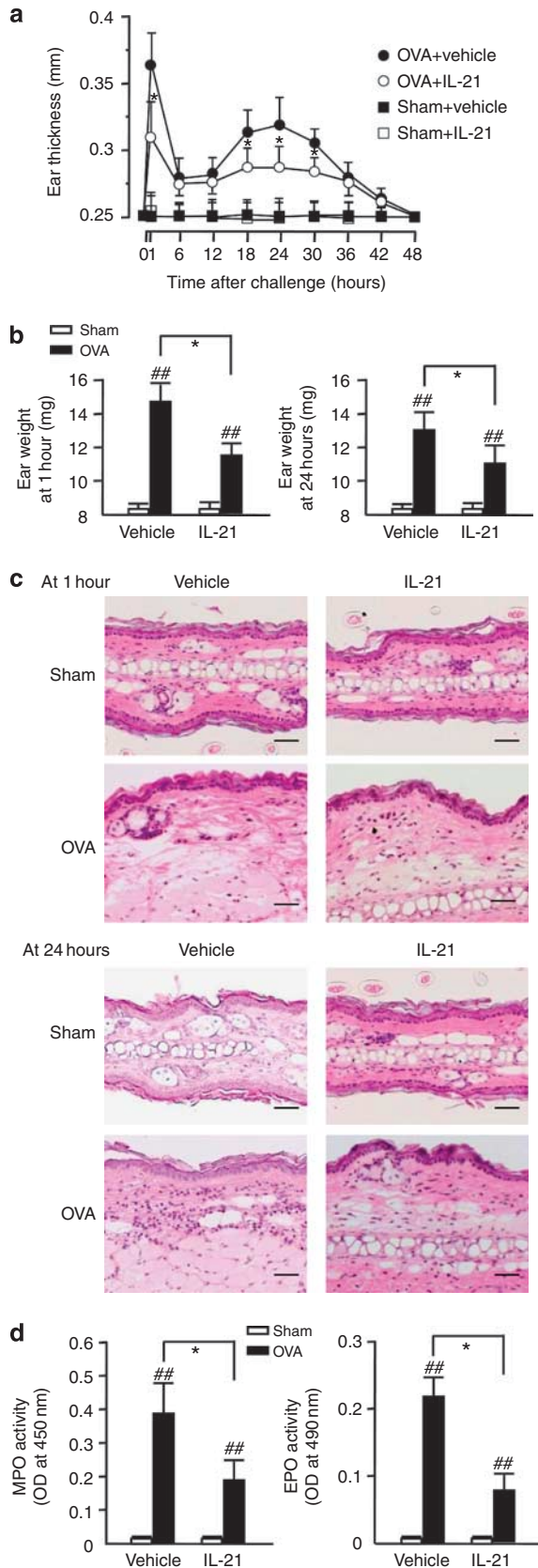
To investigate the mechanisms underlying IL-21-induced regulation of allergic cutaneous reactions, we examined the effects of rmIL-21 administration on serum IgE levels in the mouse model of the cutaneous IHR. Mice were prepared as shown in Figure 1 and serum levels of total IgE and OVA-specific IgE at challenge were evaluated. Administration of rmIL-21 during the sensitization period (Figure 1a) significantly decreased the serum levels of total IgE and OVA-specific IgE (Figure 4a). In contrast, rmIL-21 administration after completion of sensitization (Figure 1b) did not affect the amounts of total IgE and OVA-specific IgE (Figure 4b). These results suggest that during sensitization rmIL-21 can suppress the cutaneous IHR by inhibiting allergen-specific IgE production, but that mechanisms underlying the regulatory effects of rmIL-21 after completion of sensitization are not related to allergen-specific IgE production.

To investigate the effects of IL-21 on B-cell function in the mouse model of the cutaneous IHR, we examined expression of the IL-21 receptor on splenic B cells and the effects of *in vitro* IL-21 administration on IgE production by splenic B cells. Flow cytometric analysis showed that splenic B cells collected from OVA-sensitized mice expressed IL-21 receptor on the cell surface (Supplementary Figure S2-a online). Addition of rmIL-21 suppressed IgE production by splenic B cells after culturing with IL-4 and lipopolysaccharide (Supplementary Figure S2-b online). These *in vitro* data are consistent with the effects on IgE production of *in vivo* rmIL-21 administration during the sensitization period.

In vivo IL-21 administration after completion of sensitization inhibits degranulation of mast cells in skin

To investigate the mechanisms underlying the regulatory effect of IL-21 on the cutaneous IHR after completion of

Figure 2. Effect on the cutaneous immediate hypersensitivity reaction (IHR) of recombinant murine (rm)IL-21 administration during sensitization. Mice were sensitized by intraperitoneal injection of ovalbumin (OVA) and given rmIL-21 or vehicle intraperitoneally twice a week for 2 weeks during the sensitization period. Reactions to OVA were elicited by intracutaneous injection of OVA in the ear on day 14. Ear swelling responses were examined by measuring the (a) thickness and (b) weight of the ears. (c) Histology of ear skin was examined at 1 and 24 hours. Hematoxylin and eosin. Original magnification $\times 100$, scale bar = 50 μm . (d) Myeloperoxidase (MPO) and eosinophil peroxidase (EPO) activities in skin tissue were examined at 24 hours. Data are expressed as means $\pm$ SD of six mice per group. * $P < 0.01$ versus vehicle administration in OVA-challenged mice. ** $P < 0.01$ versus corresponding sham-challenged mice.



sensitization, we examined the effects of rmIL-21 on degranulation of mast cells in skin. A histological examination with toluidine blue staining showed many degranulated mast cells in the dermis in control mice at 1 hour after challenge (Figure 5a). In contrast, there were few degranulated mast cells in the dermis in rmIL-21-treated allergic mice. The percentage of degranulated mast cells was significantly lower in rmIL-21-treated allergic mice compared with controls, whereas there was no difference in the total number of dermal mast cells (Figure 5b). These results show that rmIL-21 suppresses degranulation of mast cells in skin.

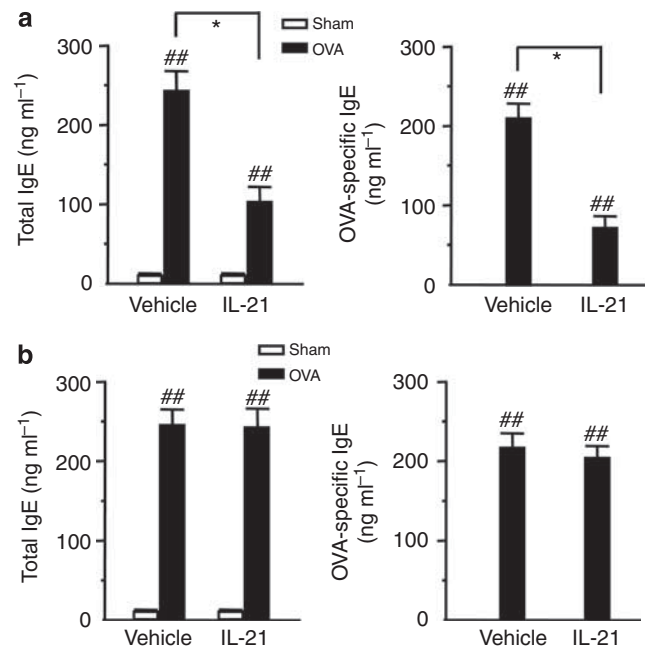


Figure 4. Serum levels of allergen-specific IgE and total IgE. (a) Mice were sensitized by intraperitoneal injection of ovalbumin (OVA) and given recombinant murine (rm)IL-21 or vehicle intraperitoneally twice a week for 2 weeks during the sensitization period. Sera were collected on day 14. (b) In separate experiments, mice were sensitized by intraperitoneal injection of OVA and given rmIL-21 or vehicle intraperitoneally once a day on days 14–17. Sera were collected at 2 hours after the final rmIL-21 administration on day 17. Serum levels of total IgE and OVA-specific IgE were measured using the corresponding ELISA kits. Data are expressed as means $\pm$ SD of six mice per group. * P < 0.01 versus vehicle in OVA-challenged mice. ** P < 0.01 versus corresponding sham-sensitized mice.

Figure 3. Effect on the cutaneous immediate hypersensitivity reaction (IHR) of recombinant murine (rm)IL-21 administration after sensitization. Mice were sensitized by intraperitoneal injection of ovalbumin (OVA) and then given rmIL-21 or vehicle intraperitoneally once a day on days 14–17. Reactions to OVA were elicited by intracutaneous injection of OVA in the ear after the final rmIL-21 administration on day 17. Ear swelling responses were determined by the (a) ear thickness and (b) weight. (c) Histology of ear skin was examined at 1 and 24 hours. Hematoxylin and eosin. Original magnification $\times$ 100, scale bar = 50 μ m. (d) Myeloperoxidase (MPO) and eosinophil peroxidase (EPO) activities in skin tissue were examined at 24 hours. Data are expressed as means $\pm$ SD of six mice per group. * P < 0.01 versus vehicle in OVA-challenged mice. ** P < 0.01 versus corresponding sham-challenged mice.

Recent reports have shown that IL-21 promotes CD8⁺ T-cell responses (Moroz *et al.*, 2004; Casey and Mescher, 2007). Therefore, to investigate the effects of IL-21 on CD8⁺ T-cell responses in the mouse model of the cutaneous IHR, we performed CD8 staining in immunohistological analysis. The number of CD8⁺ T cells in skin reduced in parallel to a decrease in the total number of skin-infiltrating cells in rmIL-21-treated allergic mice compared with controls at 24 hours after challenge (Supplementary Table S2 online), suggesting that the mechanism underlying the suppressive effects of rmIL-21 after completion of sensitization is not related to CD8⁺ T-cell responses.

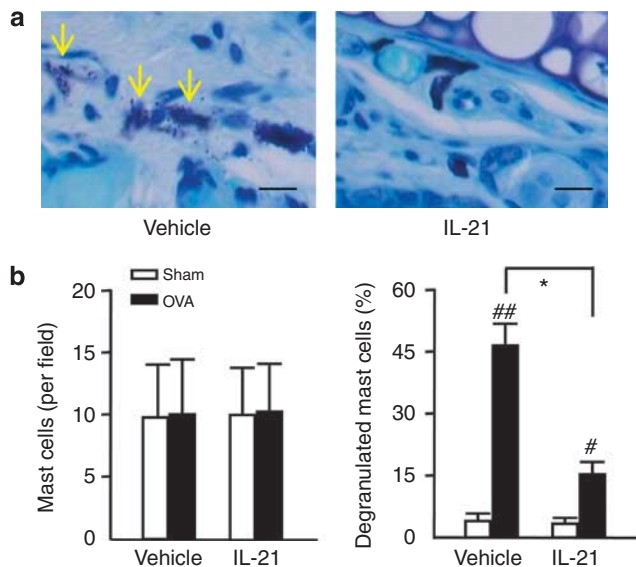
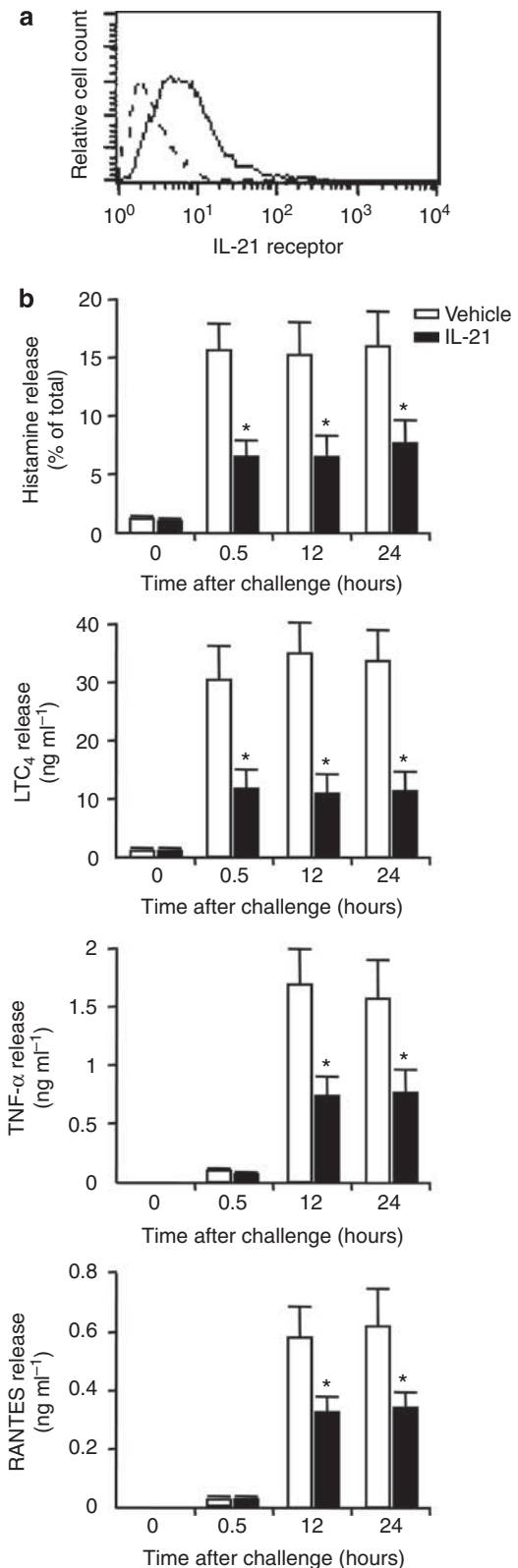


Figure 5. Effect of *in vivo* recombinant murine (rm)IL-21 administration on mast cell degranulation. (a) Mice were sensitized by injecting ovalbumin (OVA) intraperitoneally and given rmIL-21 or vehicle intraperitoneally once a day on days 14–17. Reactions to OVA were elicited by intracutaneous injection of OVA in the ear of mice at 2 hours after the final rmIL-21 administration on day 17. Ears were excised at 1 hour after challenge and samples were stained with toluidine blue. Degranulated mast cells are indicated by arrows. Original magnification $\times 400$, scale bar = 25 μ m. (b) Dermal mast cells were evaluated by averaging the number of cells present in 10 high-power fields. Data are expressed as means $\pm$ SD of six mice per group. * $P < 0.01$ versus vehicle in OVA-challenged mice. * $P < 0.05$ or ** $P < 0.01$ versus corresponding sham-challenged mice.

Figure 6. Effect of *in vitro* recombinant murine (rm)IL-21 administration on mediator release from mast cells. (a) Bone marrow-derived mast cells (BMMCs) were labeled by a specific antibody to IL-21 receptor and then IL-21 receptor expression on the surface of BMMCs was analyzed by FACS. Representative data from one of three independent experiments are shown. (b) In separate experiments, BMMCs were sensitized with anti-dinitrophenyl (DNP) IgE, incubated in the presence of rmIL-21 or vehicle, and then challenged with DNP-bovine serum albumin for 0, 0.5, 12, and 24 hours. Histamine, leukotriene (LT)_{C₄}, tumor necrosis factor- α (TNF- α), and RANTES (regulated on activation, normal T-cell expressed, and presumably secreted) levels were quantified in cell-free supernatants using the corresponding ELISA kits ($n = 3$ –5 in each group). * $P < 0.01$ versus vehicle at the same time point after challenge.

***In vitro* IL-21 administration inhibits mediator release from bone marrow-derived mast cells (BMMCs)**

To investigate whether IL-21 directly affects degranulation of mast cells, we examined the expression of the IL-21 receptor



on murine mast cells and the effects of *in vitro* IL-21 administration on mediator release from mast cells. BMMCs were generated by culturing bone marrow cells from BALB/c mice in the presence of IL-3. After 4 weeks of culture, >98% of the cells were identifiable as mast cells by morphological analysis with Giemsa staining and by FACS analysis of cell surface expression of c-Kit (data not shown). Flow cytometric analysis showed that BMMCs expressed IL-21 receptor on the cell surface (Figure 6a). Next, to investigate the direct effect of IL-21 on mediator release from murine mast cells, BMMCs were sensitized with monoclonal anti-dinitrophenyl IgE antibody, incubated in the presence of rmIL-21, and challenged with dinitrophenyl-bovine serum albumin. Measurement of mediator levels in cell-free supernatants showed that rmIL-21 suppressed the release of histamine and LTC₄ at 0.5, 12, and 24 hours and of TNF- α and RANTES at 12 and 24 hours from BMMCs (Figure 6b). To confirm the suppressive effects of IL-21 on mediator release from mast cells, anti-IL-21-neutralizing antibody was added to the culture medium of BMMCs before addition of rmIL-21. Anti-IL-21 treatment inhibited the suppressive effects of IL-21 on mediator release from mast cells (Supplementary Figure S3 online). These results indicate that IL-21 directly suppresses mediator release from murine mast cells. We next investigated whether IL-21 also affects non-IgE-mediated mast cell activation. Addition of rmIL-21 did not affect the release of mediators from phorbol 12-myristate 13-acetate/ionomycin-stimulated mast cells (Supplementary Figure S4 online), suggesting that the regulatory effect of IL-21 is restricted to IgE-mediated mast cell activation.

DISCUSSION

This study showed a, to our knowledge, previously unreported function of IL-21 in the cutaneous IHR in the mice. Intraperitoneal administration of rmIL-21 during the sensitization period or after completion of sensitization significantly suppressed the cutaneous IHR. Interestingly, the mechanisms of these suppressive effects of IL-21 differed depending on the timing of rmIL-21 administration. Thus, administration of rmIL-21 after completion of sensitization suppressed mast cell degranulation in skin, whereas administration of this cytokine during sensitization inhibited allergen-specific IgE production.

Our *in vitro* results suggest that exogenous IL-21 directly suppresses IgE-mediated activation of murine mast cells. The mediators released immediately after mast cell activation such as histamine and LTs have been revealed to be important in induction of vascular permeability changes, leading to a rapid increase in capillary permeability and edema formation (the early-phase reaction) (Galli *et al.*, 2005). In contrast, the mast cell-derived cytokines such as IL-4, IL-5, and TNF- α and chemokines such as RANTES develop an influx of inflammatory cells (the late-phase reaction) (Galli *et al.*, 2005). On activation, mast cells not only attract inflammatory cells by secreting chemotactic factors, but also induce maturation of Langerhans cells and dendritic cells that are localized with mast cells in atopic dermatitis lesions (Jawdat *et al.*, 2004; Kitawaki *et al.*, 2006).

Activated mast cells also present antigens to T cells as antigen-presenting cells by expressing major histocompatibility complex molecules (Mekori and Metcalfe, 1999; Henz *et al.*, 2001), and directly induce IgE synthesis via CD40/CD40 ligand interactions with B cells (Gauchat *et al.*, 1993). Therefore, IL-21 may exert its powerful effects on allergic skin inflammation by inhibiting these reactions via suppression of mast cell activation. The signaling pathways mediated by IL-21 in mast cells remain unclear. Therefore, further studies are required to address the mechanisms of the regulatory effects of IL-21 on IgE-mediated mast cell activation.

Our data also suggest that IL-21 administration during the sensitization period has an inhibitory effect on allergen-specific IgE production. These results are consistent with our previous findings in a mouse model of anaphylaxis (Kishida *et al.*, 2007). Regulation of IgE production by B cells differs in humans and mice. In experimental mouse models, a regulatory role of IL-21 on IgE production has been reported: exogenous IL-21 administration to mice was found to suppress allergen-specific IgE production by inhibiting IgE class switch recombination (Suto *et al.*, 2002; Hiromura *et al.*, 2007; Kishida *et al.*, 2007), whereas mice deficient in the IL-21 receptor have increased IgE levels (Ozaki *et al.*, 2002). In contrast, the positive effects of human IL-21 on IgE production by human B cells stimulated with anti-CD40 plus IL-4 have been shown (Ettinger *et al.*, 2005; Pene *et al.*, 2006). It has also been reported that IL-21 induces apoptosis of murine B cells, including IgE-bearing B cells, *in vitro* (Mehta *et al.*, 2003; Jin *et al.*, 2004; Konforte *et al.*, 2009). Taken together, these findings suggest that IL-21 suppresses allergen-specific IgE production by inhibiting IgE class switch recombination and/or by inducing apoptosis of B cells in mice. In this study, rmIL-21 administration after completion of sensitization did not reduce serum levels of allergen-specific IgE. Because the half-life of murine IgE in serum is relatively short (5–12 hours) (Haba *et al.*, 1985; Vieira and Rajewsky, 1988), IgE production may not be inhibited by IL-21 after generation of IgE-producing B cells. Because IL-21 induces apoptosis of B cells through nonspecific stimulation via B-cell receptors or strong signals via Toll-like receptors (Konforte *et al.*, 2009), this cytokine may fail to affect the survival of existing IgE-producing B cells under the experimental conditions in the study.

We note that there are conflicting data on the effects of IL-21 on allergic skin inflammation. It has been reported that IL-21-treated dendritic cells fail to induce hapten-induced contact hypersensitivity reactions via inhibition of dendritic cell activation and maturation *in vivo* (Brandt *et al.*, 2003). In contrast, IL-21 receptor deficiency inhibited allergic cutaneous inflammation by suppressing trafficking of cutaneous dendritic cells to draining lymph nodes in a murine model of epicutaneous sensitization using tape stripping (Jin *et al.*, 2009). In this study, we showed that exogenous IL-21 administration suppressed the cutaneous IHR, whereas anti-IL-21-neutralizing antibody treatment did not influence the cutaneous reaction. In addition, we found that exogenous IL-21 administration did not affect chronic dermatitis in another murine model of repeated hapten epicutaneous

application (Tamagawa-Mineoka *et al.*, unpublished data). These studies used a variety of allergic cutaneous inflammation protocols, and the effects of IL-21 on allergic dermatitis *in vivo* may differ depending on the inflammatory conditions of the experiments. Therefore, further studies of the various mechanisms underlying the effects of IL-21 on allergic cutaneous inflammation are required before possible clinical use of this cytokine.

In conclusion, this study revealed the *in vivo* effects of exogenous IL-21 on the cutaneous IHR. Administration of rmIL-21 during sensitization inhibited the cutaneous IHR by regulating allergen-specific IgE production, whereas rmIL-21 given after completion of sensitization suppressed mast cell degranulation in skin. Therefore, IL-21 may have potential for treatment of allergic cutaneous diseases.

MATERIALS AND METHODS

Mice

Male BALB/c mice were purchased from Shimizu Laboratory Supplies (Kyoto, Japan) and used at age 6–8 weeks. The experimental procedure was approved by the committee for animal research, Kyoto Prefectural University of Medicine.

Sensitization

The biphasic allergic murine skin model was prepared as described previously (Sawada *et al.*, 1997; Tamagawa-Mineoka *et al.*, 2009). Mice were sensitized by intraperitoneal injection of 3 µg OVA (grade V; Sigma-Aldrich, St Louis, MO) and 4 mg aluminum hydroxide (Pierce, Rockford, IL). Reactions to OVA were elicited by intracutaneous injection of 10 µg OVA in the right ear of mice on day 14 (Figure 1a) or day 17 (Figure 1b), with the same volume of vehicle injected into the left ear as a control.

IL-21 administration

Groups of mice were injected intraperitoneally with rmIL-21 (0.5 µg per mouse; R&D Systems, Minneapolis, MN) or vehicle as a control twice a week for 2 weeks during the sensitization period, with the first injection being performed 2 hours before injection of OVA for sensitization (Figure 1a). In separate experiments, groups of mice were given rmIL-21 (0.5 µg per mouse) or vehicle intraperitoneally once a day on days 14–17, with the final injection being performed 2 hours before OVA challenge (Figure 1b).

Histological examination

Ears were excised at 1 and 24 hours after challenge. Samples were fixed with 10% formalin and embedded in paraffin. Sections (5 µm) were cut using a microtome and stained with hematoxylin and eosin for general histological evaluation and toluidine blue for mast cell staining.

Measurement of MPO and eosinophil peroxidase activities

Ear tissue was homogenized and MPO activity was measured using an MPO assay kit (CytoStore, Calgary, Alberta, Canada) with absorbance measured at 450 nm. Eosinophil peroxidase activity was measured based on absorbance at 490 nm after incubation of a homogenate of ear tissue (50 µl) with 100 µl of 50 mmol l⁻¹ Tris-buffered saline containing 0.1% Triton X-100, 1 mmol l⁻¹ o-phenylenediamine, and 500 µmol l⁻¹ hydrogen peroxide, as previously described (Kawase *et al.*, 2003).

Measurement of serum IgE

Sera were collected on day 14 in both protocols shown in Figure 1 and at 2 hours after the final administration of rmIL-21 on day 17 in the protocol shown in Figure 1b. Serum levels of total IgE and OVA-specific IgE were evaluated using the corresponding ELISA kits purchased from BD PharMingen (San Diego, CA) and Dainippon Sumitomo Pharma (Osaka, Japan), respectively.

Preparation of BMMCs

BMMCs were generated from femoral bone marrow cells of male BALB/c mice using a previously described method (Nakano *et al.*, 1985) with slight modifications. Bone marrow cells were cultured in RPMI-1640 (GIBCO, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum, 100 U ml⁻¹ penicillin, 100 µg ml⁻¹ streptomycin, 100 µM 2-mercaptoethanol, 1 mM sodium pyruvate, 0.1 mM minimal essential medium with nonessential amino acid solution, and 10 ng ml⁻¹ rmIL-3 (Peprotech, Rocky Hill, NJ) at 37 °C for 4 weeks.

Flow cytometry

Cells were incubated with rat anti-mouse IL-21 receptor antibody (clone 155516; R&D Systems) for 30 minutes at 4 °C. After washing, the samples were incubated with secondary FITC-labeled goat anti-rat IgG. Following further washing, the cells were analyzed by FACS (Becton Dickinson, San Jose, CA). Phycoerythrin-labeled rat anti-mouse CD117 antibody (clone 180627; R&D Systems) was used in FACS analysis of c-Kit expression on BMMCs.

Stimulation of BMMCs

BMMCs were stimulated using a previously described method (Malaviya and Uckun, 2002) with slight modifications. BMMCs were sensitized with monoclonal anti-dinitrophenyl IgE antibody (0.24 mg ml⁻¹; clone SPE-7; Sigma-Aldrich) overnight at 37 °C. Unbound IgE was removed by washing the cells twice with phosphate-buffered saline. After washing, the BMMCs were resuspended in RPMI-1640 at a cell density of 1 × 10⁶ per ml and cultivated for 60 minutes in the presence of 40 ng ml⁻¹ rmIL-21. The cells were then challenged with 20 ng ml⁻¹ dinitrophenyl-bovine serum albumin (LSL, Tokyo, Japan) for 0, 0.5, 12, and 24 hours at 37 °C.

Mediator release assays

Quantitative determination of histamine, LTC₄, TNF-α, and RANTES in cell-free supernatants was performed with a histamine enzyme immunoassay kit (SPI-BIO, Bretonneux, France), mouse LTC₄ ELISA kit (Cusabio Biotech, Newark, DE), mouse TNF-α ELISA kit (Abnova, Taoyuan, Taiwan), and mouse RANTES ELISA kit (Peprotech), respectively.

Statistical analysis

Data from all studies are expressed as means ± SD. Statistical significance was assessed by one-way analysis of variance followed by Tukey's multiple comparison test. Student's *t*-test was used for comparison of mediator release from BMMCs. A *P*-value of <0.05 was considered significant.

Complete Materials and Methods and associated references are available in the Supplementary Information online.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

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

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Supplementary Information

IL-21 reduces immediate hypersensitivity reactions in mouse skin by suppressing mast cell activation or IgE production

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Complete Materials and Methods

Mice

Male BALB/c mice were purchased from Shimizu Laboratory Supplies (Kyoto, Japan) and used at age 6 to 8 weeks. All mice were housed in a specific pathogen-free barrier facility and screened regularly for pathogens. The experimental procedure was approved by the Committee for Animal Research, Kyoto Prefectural University of Medicine.

Sensitization

The biphasic allergic murine skin model was prepared as described previously (Sawada et al, 1997; Tamagawa-Mineoka et al, 2009). Mice were sensitized by intraperitoneal injection of 3 µg OVA (grade V; Sigma-Aldrich, St. Louis, MO) and 4 mg aluminum hydroxide (Pierce, Rockford, IL) in a volume of 0.2 ml/mouse. Reactions to OVA were elicited by intracutaneous injection of 10 µg OVA in a 20-µl volume using a 30-gauge needle in the right ear of mice on day 14 (Figure 1a) or day 17 (Figure 1b), with the same volume of vehicle injected into the left ear as a control. The ear swelling response was assessed by measuring ear thickness and weight. Ear thickness was measured after 1 hour and every 6 hours after challenge. At 1 hour and 24 hours after challenge, a 5-mm diameter biopsy of the ear was taken and weighed.

IL-21 administration

Groups of mice were injected intraperitoneally with rmIL-21 (0.5 µg/mouse; R & D Systems, Minneapolis, MN) or vehicle as a control twice a week for two weeks during the sensitization period, with the first injection being performed 2 hours before injection of OVA for sensitization (Figure 1a). In separate experiments, groups of mice were given rmIL-21 (0.5 µg/mouse) or vehicle intraperitoneally once a day on days 14-17, with the final injection being performed 2 hours before OVA challenge (Figure 1b). The injection of rmIL-21 did not affect the general condition of the mice.

In vivo anti-IL-21-neutralizing antibody treatment

Sensitized mice were challenged by injecting intradermally with OVA plus

anti-IL-21-neutralizing antibody (50 µg) (R&D Systems) or isotype-matched control IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) on day 14.

Histological examination

Ears were excised at 1 hour and 24 hours after challenge. Samples were fixed with 10% formalin and embedded in paraffin. Sections (5 µm) were cut using a microtome and stained with hematoxylin and eosin for general histological evaluation and toluidine blue for mast cell staining. Dermal mast cells were evaluated by averaging the number of cells present in 10 high-power fields. Degranulation of mast cells was judged by the extrusion of granule content and/or >50% loss of cellular staining using the criteria of Theoharides et al (1998). The percentage degranulation was calculated by dividing the number of degranulated mast cells by the total number of mast cells.

For immunohistochemistry, samples were frozen in OCT compound (Sakura Fine Technical, Tokyo, Japan) and cut using a microtome. Sections (4 µm) were incubated with goat polyclonal anti-IL-21 antibody (80 mg/ml; R&D Systems) or goat polyclonal anti-CD8 antibody (50 µg/ml; Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C. Sections were then incubated sequentially with biotinylated rabbit anti-goat IgG and horseradish peroxidase-conjugated avidin-biotin. Sections were developed with 3,3'-diaminobenzidine tetrahydrochloride and hydrogen peroxidase and then counterstained with hematoxylin. Cutaneous expression of IL-21 was scored semiquantitatively (0, no expression; 1, weak expression; 2, moderate expression; 3, strong expression) as previously described (Wenzel et al, 2005; Jin et al, 2009). Dermal CD8⁺ cells were evaluated by averaging the number of cells present in 10 high-power fields.

Measurement of MPO and EPO activities

Ear tissue was homogenized and MPO activity was measured using an MPO assay kit (CytoStore, Calgary, AB, Canada) with absorbance measured at 450 nm. EPO activity was measured based on absorbance at 490 nm after incubation of a homogenate of ear tissue (50 µl) with 100 µl of 50 mmol/l Tris-buffered saline containing 0.1% Triton X-100, 1 mmol/l o-phenylenediamine, and 500 µmol/l hydrogen peroxide, as previously described (Kawase et al, 2003).

Measurement of serum IgE

Sera were collected on day 14 in both protocols shown in Figure 1 and at 2 hours after

the final administration of rmIL-21 on day 17 in the protocol shown in Figure 1b. Serum levels of total IgE and OVA-specific IgE were evaluated using the corresponding ELISA kits purchased from BD PharMingen (San Diego, CA) and Dainippon Sumitomo Pharma (Osaka, Japan), respectively.

Preparation of BMMCs

BMMCs were generated from femoral bone marrow cells of male BALB/c mice using a previously described method (Nakano et al, 1985) with slight modifications. Bone marrow cells were cultured in RPMI 1640 (GIBCO, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 100 µM 2-mercaptoethanol, 1 mM sodium pyruvate, 0.1 mM minimal essential medium with nonessential amino acid solution, and 10 ng/ml rmIL-3 (Peprotech, Rocky Hill, NJ) at 37°C in a humidified atmosphere with 5% CO₂. Nonadherent cells were transferred to fresh medium every 2-3 days for 4 weeks to remove adherent macrophages and fibroblasts.

Flow cytometry

Cells were incubated with rat anti-mouse IL-21 receptor antibody (clone 155516; R & D Systems) for 30 minutes at 4°C. After washing, the samples were incubated with secondary fluorescein isothiocyanate-labeled goat anti-rat IgG. Following further washing, the cells were analyzed by FACS (Becton Dickinson, San Jose, CA). Phycoerythrin-labeled rat anti-mouse CD117 antibody (clone 180627; R & D Systems) was used in FACS analysis of c-Kit expression on BMMCs.

Stimulation of BMMCs

BMMCs were stimulated using a previously described method (Malaviya and Uckun, 2002) with slight modifications. BMMCs were sensitized with monoclonal anti-DNP IgE antibody (0.24 mg/ml; clone SPE-7; Sigma-Aldrich) overnight at 37°C. Unbound IgE was removed by washing the cells twice with phosphate-buffered saline. After washing, the BMMCs were resuspended in RPMI 1640 at a cell density of 1×10^6 /ml and cultivated for 60 minutes in the presence of 40 ng/ml rmIL-21. The cells were then challenged with 20 ng/ml DNP-bovine serum albumin (LSL Co., Tokyo, Japan) for 0, 0.5, 12, and 24 hours at 37°C. Supernatants were removed and frozen at -80°C until analysis. Alternatively, BMMCs were left unsensitized and stimulated with 100 nM phorbol 12-myristate 13-acetate (Sigma-Aldrich) and 100 nM ionomycin (Wako Pure Chemical Industries, Osaka, Japan).

In vitro treatment with anti-IL-21-neutralizing antibody, goat anti-IL-21-neutralizing antibody (20 µg/ml; R&D Systems) or isotype-matched control IgG (Jackson ImmunoResearch Laboratories) was added to the culture medium prior to addition of rmIL-21. Bound antibodies were removed using magnetic activated cell sorting with protein G magnetic beads (Miltenyi Biotec) according to the manufacture's protocol.

Mediator release assays

Quantitative determination of histamine, LTC₄, TNF-α, and RANTES in cell-free supernatants was performed with a histamine enzyme immunoassay kit (SPI-BIO, Bretonneux, France), mouse LTC₄ ELISA kit (Cusabio Biotech, Newark, DE), mouse TNF-α ELISA kit (Abnova, Taoyuan, Taiwan), and mouse RANTES ELISA kit (Peprotech, Rocky Hill, NJ), respectively.

IgE production from cultured splenic B cells

Spleens from male BALB/c mice were homogenized in RPMI 1640 medium. Purified spleen cells were separated into enriched populations of B cells using magnetic activated cell sorting with anti-B220 antibody-coated magnetic beads (Miltenyi Biotec). Splenic B cells were stimulated with lipopolysaccharide (10 µg/ml; Sigma-Aldrich) in the presence of rmIL-4 (50 ng/ml; R & D Systems) and rmIL-21 (40 ng/ml; R & D Systems) or vehicle in RPMI 1640 medium at 37°C for 7 days. The amount of total IgE in the supernatant was measured using the corresponding ELISA kit (Yamasa, Chiba, Japan).

Statistical analysis

Data from all studies are expressed as means ± SD. Statistical significance was assessed by one-way analysis of variance followed by a Tukey multiple comparison test. A student t test was used for comparison of mediator release from BMMCs, number and proportion of CD8⁺ T cells, and cutaneous expression score of IL-21. A P value of less than 0.05 was considered significant.

References for the supplemental methods

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Supplementary Tables

Table S1. Serum levels of total IgE and anti-OVA IgE after sensitization

	OVA-sensitized mice	Sham-sensitized mice
Total IgE (ng/ml)	242 $\pm$ 17	10.6 $\pm$ 1.1
Anti-OVA IgE (ng/ml)	216 $\pm$ 15	not detected

Data are expressed as the means $\pm$ SD of 6 mice per group.

Table S2. Number and proportion of CD8⁺ T cells in inflamed skin

	IL-21-treated mice	Control mice
Number of CD8 ⁺ T cells (/field)	5.5 ± 1.0*	10.8 ± 2.3
Proportion of CD8 ⁺ T cells among skin-infiltrating cells (%)	5.4 ± 1.3	5.3 ± 1.1

Data are expressed as means ± SD of 6 mice per group.

**P*<0.01 versus vehicle-treated control mice.

Supplementary Figures

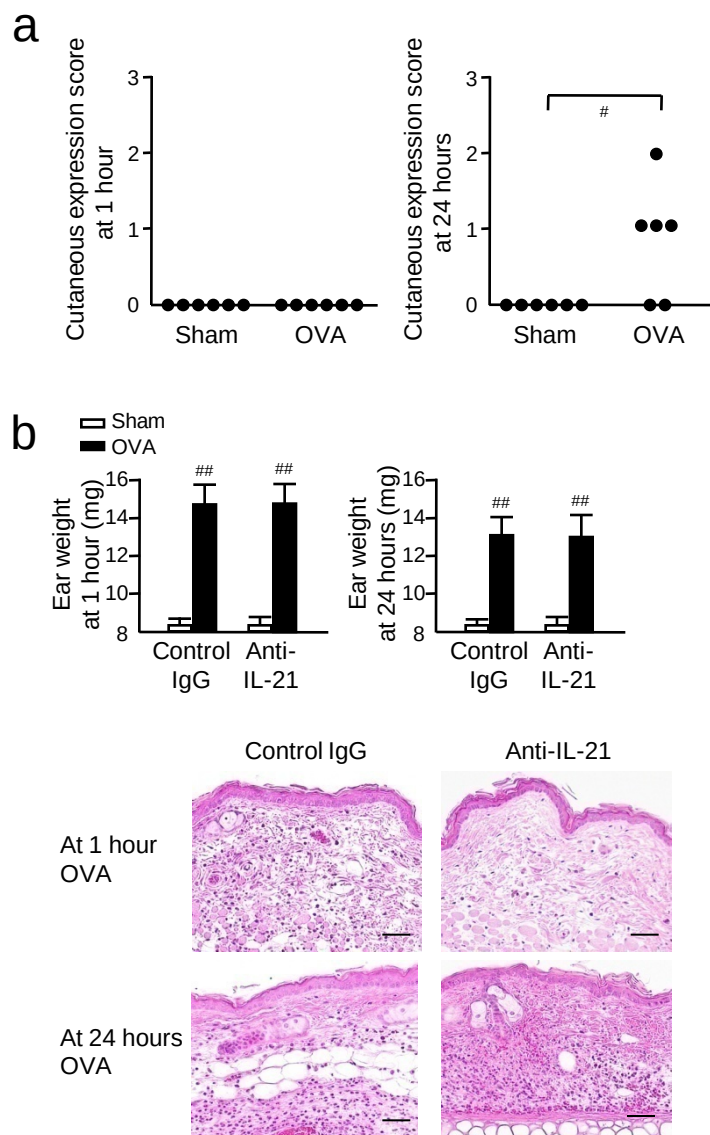


Figure S1. Expression of IL-21 in inflamed skin and effect of anti-IL-21-neutralizing antibody treatment on the cutaneous IHR. Mice were sensitized intraperitoneally with OVA, and then challenged with OVA or vehicle on day 14. Immunohistological analysis of ear skin was performed at 1 and 24 hours after challenge using anti-IL-21 antibody. Cutaneous expression of IL-21 in skin in OVA- or sham-challenged mice was scored semiquantitatively as described in Supplementary Materials and Methods (a). In separate experiments, sensitized mice were challenged by injecting intradermally with OVA plus anti-IL-21-neutralizing antibody or isotype-matched control IgG on day 14 (b). Ear swelling responses were determined by the ear weight. Histology of ear skin was examined at 1 and 24 hours. Hematoxylin and eosin. Original magnification $\times 100$, bar=50 μm . Data are expressed as means $\pm$ SD of 6 mice per group. # $P < 0.05$ or ## $P < 0.01$ versus corresponding sham-challenged mice.

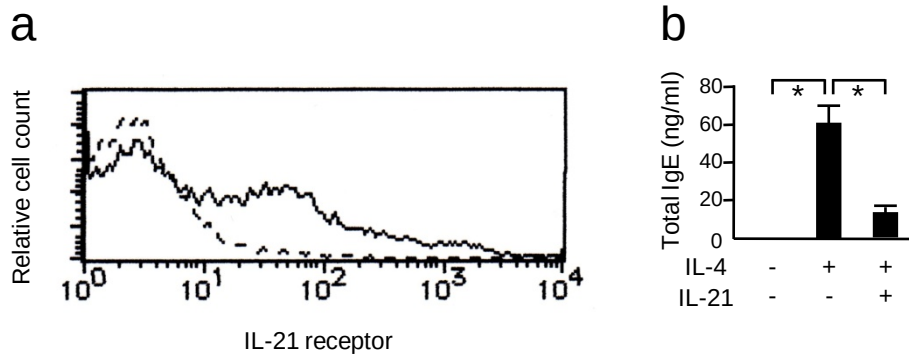


Figure S2. Effect of IL-21 on B cell function in the mouse model of the cutaneous IHR. Purified splenic B cells were labeled by a specific antibody to IL-21 receptor and then IL-21 receptor expression on the surface of splenic B cells was analyzed by FACS (a). Representative data from one of three independent experiments are shown. In separate experiments, splenic B cells were stimulated by lipopolysaccharide with/without rmIL-4 and rmIL-21 for 7 days. IgE levels in the supernatant of the culture were determined (n=3 in each group) (b). * $P<0.01$.

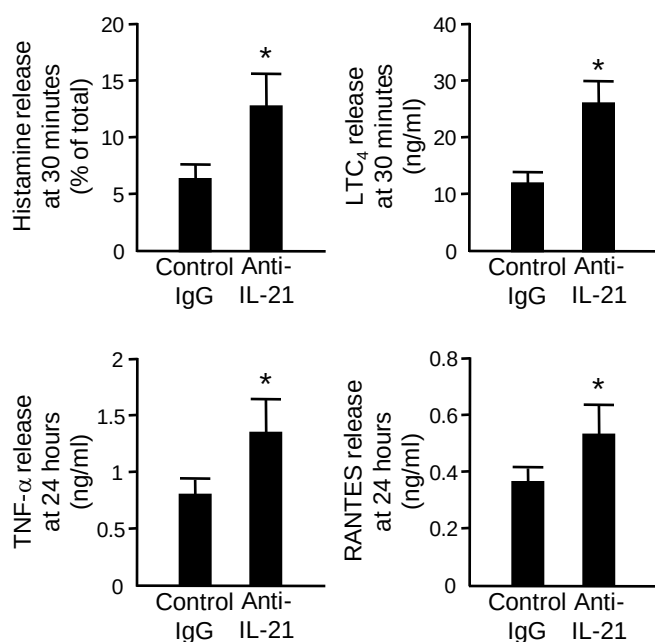


Figure S3. Effect of IL-21 on the release of mediators by anti-IL-21-treated mast cells. BMMCs were sensitized with anti-DNP IgE, and anti-IL-21-neutralizing antibody or isotype-matched control IgG was added to the culture medium of BMMCs. And then BMMCs were incubated with an addition of rmIL-21 and challenged with DNP-bovine serum albumin for 30 minutes or 24 hours. Histamine, LTC₄, TNF- α , and RANTES levels were quantified in cell-free supernatants using the corresponding ELISA kits (n=5 in each group). * P <0.01.

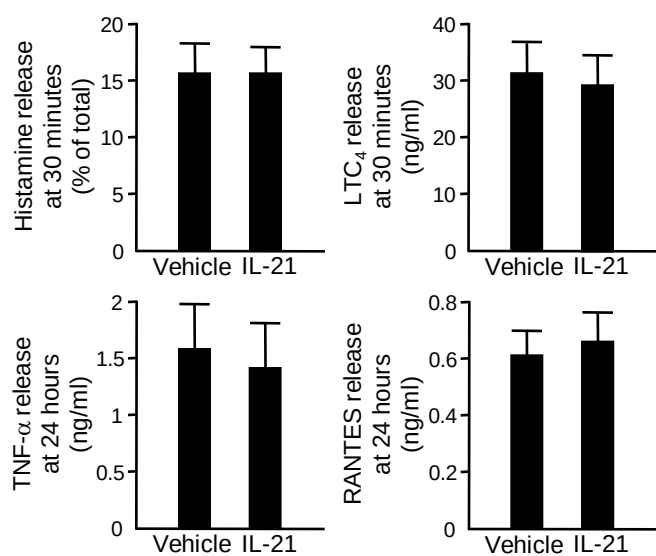


Figure S4. Effect of IL-21 on non-IgE-mediated mast cell activation. BMMCs were incubated in the presence of rmIL-21 or vehicle, and then stimulated with phorbol 12-myristate 13-acetate and ionomycin for 30 minutes or 24 hours. Histamine, LTC₄, TNF- α , and RANTES levels were quantified in cell-free supernatants using the corresponding ELISA kits (n=5 in each group).