

# IL-21R is essential for epicutaneous sensitization and allergic skin inflammation in humans and mice

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**Atopic dermatitis (AD) is a common allergic inflammatory skin disease caused by a combination of intense pruritus, scratching, and epicutaneous (e.c.) sensitization with allergens. To explore the roles of IL-21 and IL-21 receptor (IL-21R) in AD, we examined skin lesions from patients with AD and used a mouse model of allergic skin inflammation. IL-21 and IL-21R expression was upregulated in acute skin lesions of AD patients and in mouse skin subjected to tape stripping, a surrogate for scratching. The importance of this finding was highlighted by the fact that both *Il21r*<sup>-/-</sup> mice and WT mice treated with soluble IL-21R-IgG2aFc fusion protein failed to develop skin inflammation after e.c. sensitization of tape-stripped skin. Adoptively transferred OVA-specific WT CD4<sup>+</sup> T cells accumulated poorly in draining LNs (DLNs) of e.c. sensitized *Il21r*<sup>-/-</sup> mice. This was likely caused by both DC-intrinsic and nonintrinsic effects, because trafficking of skin DCs to DLNs was defective in *Il21r*<sup>-/-</sup> mice and, to a lesser extent, in WT mice reconstituted with *Il21r*<sup>-/-</sup> BM. More insight into this defect was provided by the observation that skin DCs from tape-stripped WT mice, but not *Il21r*<sup>-/-</sup> mice, upregulated CCR7 and migrated toward CCR7 ligands. Treatment of epidermal and dermal cells with IL-21 activated MMP2, which has been implicated in trafficking of skin DCs. These results suggest an important role for IL-21R in the mobilization of skin DCs to DLNs and the subsequent allergic response to e.c. introduced antigen.**

## Introduction

IL-21, a pleiotropic cytokine derived from activated CD4<sup>+</sup> T cells and NKT cells (1, 2), belongs to the family of common  $\gamma$  ( $\gamma_c$ ) chain-dependent cytokines. The IL-21 receptor (IL-21R) consists of the IL-21R $\alpha$  chain and the  $\gamma_c$  chain and is expressed on T cells, NK cells, NKT cells, B cells, DCs, and macrophages as well as on non-hematopoietic cells, including keratinocytes and fibroblasts (3).

IL-21 has previously been reported to inhibit IFN- $\gamma$  production by developing Th1 cells (4) and to synergize with IL-15 to enhance the production of IFN- $\gamma$  by T cells (5, 6). However, Th1 cytokine production after anti-CD3 stimulation in vitro or antigen immunization in vivo is intact in *Il21r*<sup>-/-</sup> mice (7). *Il21r*<sup>-/-</sup> mice have normal numbers of Th2 cytokine-producing T cells and normal cytokine production in the spleen in response to parasitic infection and i.p. immunization with antigen (8, 9). However, following airway antigen challenge, Th2 cells accumulate poorly in the lungs, but normally in draining LNs (DLNs), of *Il21r*<sup>-/-</sup> mice

(8, 9). This is associated with impaired granuloma formation in response to parasitic infection and decreased airway inflammation in response to antigen inhalation challenge. IL-21 cooperates with TGF- $\beta$  to promote the generation of Th17 cells independently of IL-6 (10–12). IL-21 promotes the expansion and maturation of NK cells and enhances their cytolytic function against a wide spectrum of targets (13–15). IL-21 produced by NKT cells enhances, in an autocrine fashion, NKT cell survival and proliferation as well as cytokine production (2). IL-21 promotes B cell differentiation by synergizing with BAFF and enhancing CD40 induction of activation-induced deaminase and Blimp1 (16). However, IL-21 inhibits IgE production by inhibiting C $\epsilon$  germline transcription and IgG1<sup>+</sup> B cells from switching to IgE (17). Consequently, *Il21r*<sup>-/-</sup> mice have elevated levels of IgE but decreased levels of other Ig isotypes (7).

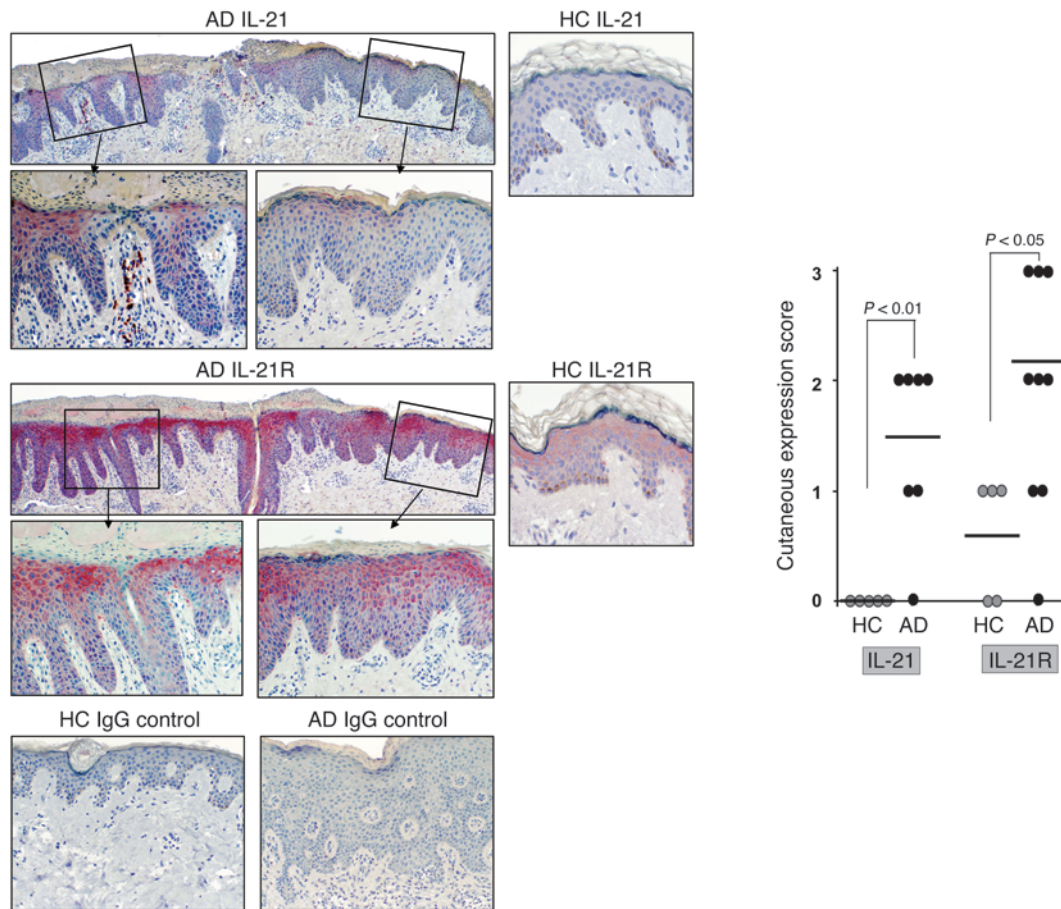
Atopic dermatitis (AD) is a pruritic allergic inflammatory skin disease that affects more than 15% of children (18). Acute AD skin lesions are characterized by epidermal and dermal thickening, by dermal infiltration of CD4<sup>+</sup> T cells and eosinophils, and by local expression of Th2 cytokines (19). Epicutaneous (e.c.) sensitization with allergens plays an important role in the pathogenesis of AD. Approximately 80% of patients with AD are sensitized to allergens, as evidenced by elevated serum total IgE levels with specific IgE antibodies to environmental and/or food allergens (19). Application of allergens to the abraded uninvolved skin of patients with AD provokes an eczematous rash with eosinophilic infiltration (20). A hallmark of AD is dry, itchy skin, likely caused by defects in skin genes that are important for maintaining skin barrier

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**Conflict of interest:** Mary Collins is an employee of Wyeth Research. Deborah Young is an employee of and owns stock options in Wyeth Research. Hans C. Oettgen is a consultant for Schering-Plough Corporation and receives research support from Novartis Pharmaceuticals Corporation.

**Nonstandard abbreviations used:** AD, atopic dermatitis; BMDC, BM-derived DC; BMMC, BM-derived mast cell; DLN, draining LN; e.c., epicutaneous (ly); i.d., intradermal (ly); IL-21R, IL-21 receptor; IL-21R:Fc, soluble IL-21R-IgG2aFc fusion protein; TSLP, thymocyte stromal lymphopoietin.

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**Figure 1** IL-21 and IL-21R expression is upregulated in skin lesions of acute AD patients and following mechanical skin injury. IL-21 and IL-21R protein expression in skin from a healthy control (HC) and in an acute skin lesion from a patient with AD. Original magnification,  $\times 200$ ;  $\times 400$  (higher-magnification panels). For the patient with AD, the left side of the biopsy shows a more active skin lesion than does the right side. Cutaneous expression of IL-21 and IL-21R in 7–9 AD patients and 5 healthy controls was scored semiquantitatively as described in Methods. Each point represents a patient; horizontal bars denote means.

function and turgidity (21). More than 10% of patients with AD have a heterozygous loss-of-function mutation in the epidermally expressed filaggrin gene, which is important for skin barrier function (22). Intense pruritus and the resulting scratching cause mechanical skin injury, which further increases skin permeability to allergens and leads to the release of cytokines and chemokines, promoting a Th2-dominated allergic response (23).

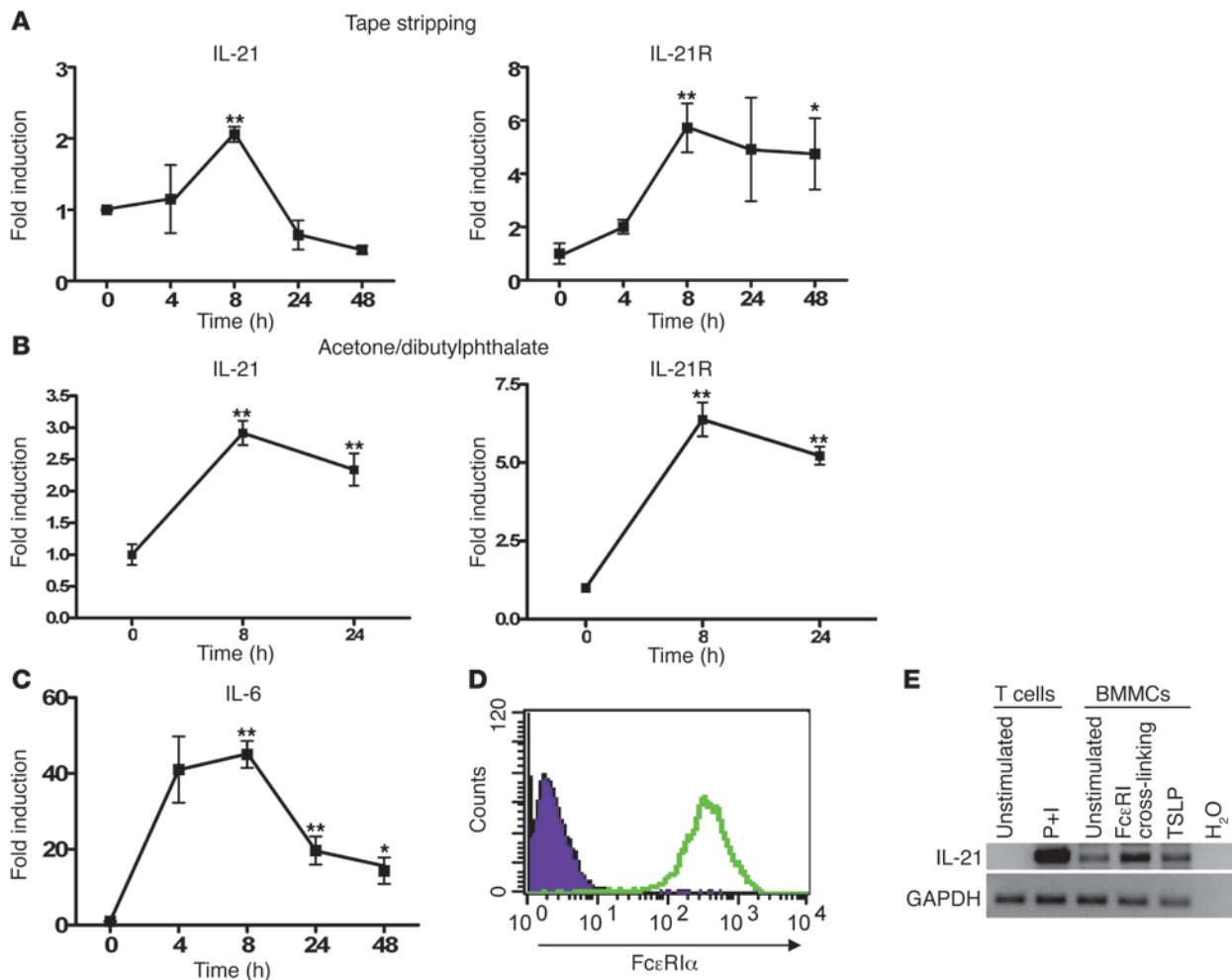
Prompted by the observation that IL-21 and IL-21R are highly expressed in skin lesions of AD patients, we used a mouse model of allergic skin inflammation elicited by repeated e.c. application of OVA to tape-stripped skin, which mimics many features of AD (24, 25), to examine the role of IL-21/IL-21R interactions in the inflammatory response to e.c. introduced antigen. We demonstrate that IL-21/IL-21R interactions play a critical role in allergic skin inflammation to e.c. introduced antigen by promoting the migration of skin DCs to DLNs and the subsequent elicitation of an immune response.

**Results**

*IL-21 and IL-21R protein expression is upregulated in skin lesions of human AD.* Expression of IL-21 and IL-21R protein was examined by immunohistochemistry in skin lesions from patients with active AD

and in skin from healthy controls. Both proteins were evident in AD skin lesions. IL-21 was expressed by mononuclear leukocytes that infiltrate the dermis and was weakly detected in the epidermis of AD skin lesions, particularly in areas with more severe acute eczematous changes (Figure 1). IL-21R expression was observed not only in epidermal keratinocytes of AD skin lesions, but also along the dermal/epidermal junction in a granular expression pattern. In contrast, IL-21 protein expression was not detected in normal skin, and only weak IL-21R expression was seen. Cutaneous expression of IL-21 and IL-21R was scored semiquantitatively as described in Methods, and statistical analysis revealed significantly increased expression of IL-21 and IL-21R protein in AD skin lesions compared with normal skin (Figure 1).

*IL-21 and IL-21R are expressed in normal mouse skin, and their expression is upregulated by tape stripping.* Mechanical injury induced by scratching dry, itchy skin underlies human AD. We asked whether mechanical injury upregulates IL-21 and IL-21R expression in normal skin. Tape stripping of mouse back skin was used to mimic mechanical injury inflicted by scratching. Figure 2A shows that *Il21* mRNA levels were upregulated 2-fold in mouse skin 8 h after tape stripping. Tape stripping caused robust and sustained upregulation

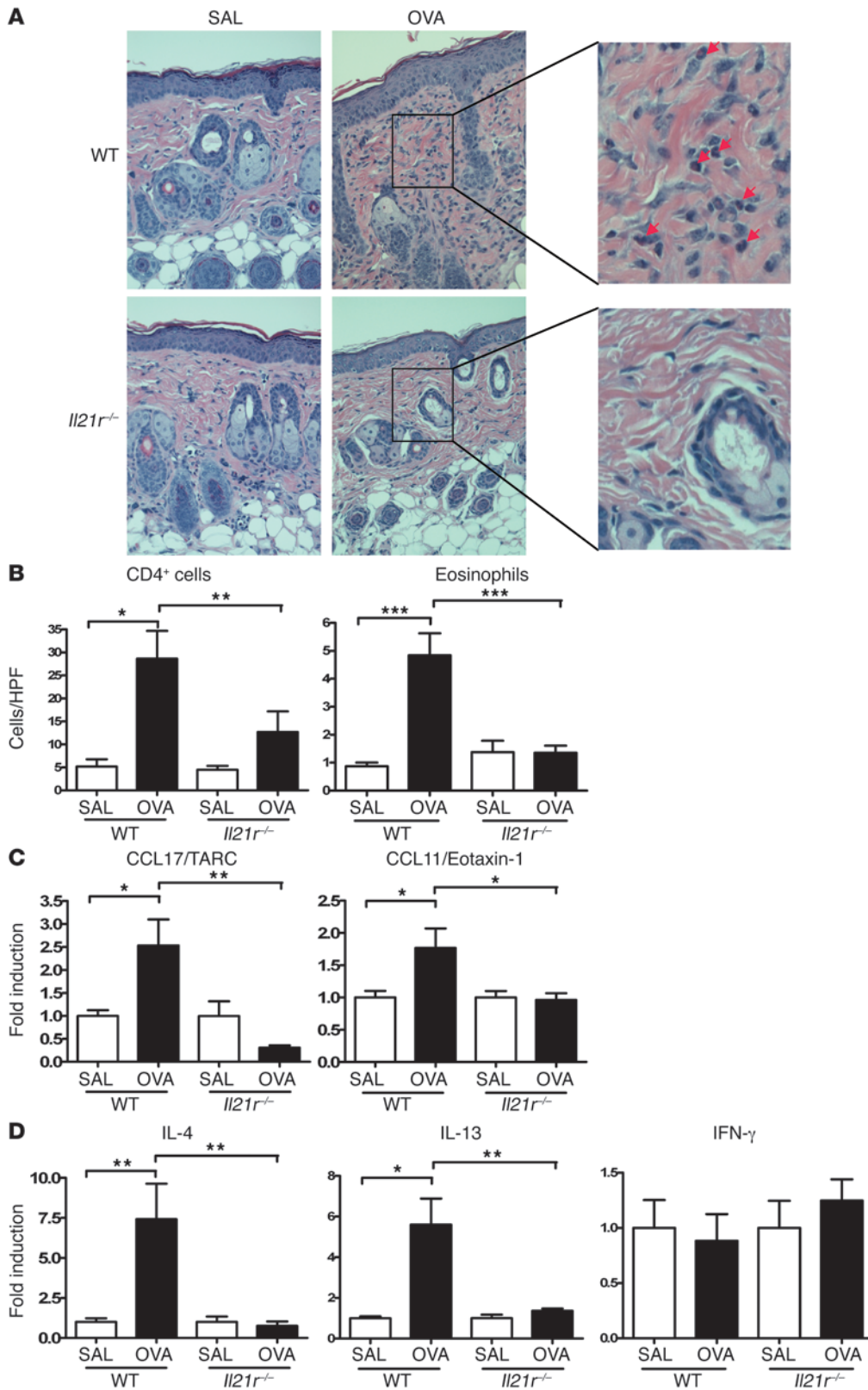
**Figure 2**

IL-21 and IL-21R expression is upregulated after mechanical skin injury. (A and B) Quantitative RT-PCR analysis of *Il21* and *Il21r* mRNA expression in mouse skin following tape stripping (A) or application of acetone/dibutylphthalate to shaved skin (B). Results were normalized for *Gapdh* expression. (C) Time course of *Il6* mRNA expression in tape-stripped skin. (D) FACS analysis of BMMCs for FcεRIα expression (open histogram). Filled histogram represents staining with isotype control. (E) RT-PCR analysis of *Il21* mRNA expression in splenic CD4<sup>+</sup> T cells and BMMCs. T cells were stimulated with P+I; BMMCs were stimulated with 0.25 μg/ml IgE anti-DNP plus 30 ng/ml DNP-HSA (cross-linking) or with 50 ng/ml TSLP for 24 h. Water was used as negative control. Data in A–C are expressed as fold induction compared with time point 0 h. Values are mean ± SD. \**P* < 0.05, \*\**P* < 0.01 versus 0 h. *n* = 5 per group.

of *Il21r* mRNA levels in skin (Figure 2A). There was also modest, but significant, 3-fold upregulation of *Il21* mRNA levels, and robust upregulation of *Il21r* mRNA levels, in shaved skin after painting with a 1:1 ratio of acetone/dibutylphthalate, the vehicle used for FITC sensitization (Figure 2B). IL-21 is expressed by CD4<sup>+</sup> T cells after antigen receptor stimulation and also after IL-6 stimulation (10–12). Figure 2C shows that tape stripping strongly upregulated IL-6 expression in the skin. CD4<sup>+</sup> cells constitute a minority of skin cells; however, the skin is rich in mast cells. We found that *Il21* mRNA was expressed in BM-derived mast cells (BMMCs), which consisted of greater than 99% FcεRI<sup>+</sup> cells (Figure 2, D and E). Mast cells are activated by cross-linking of their high-affinity IgE receptor FcεRI, but also by the keratinocyte-derived cytokine thymocyte stromal lymphopoietin (TSLP). TSLP expression in skin is upregulated by mechanical injury and by painting shaved skin with acetone/dibutylphthalate (S. Ziegler, unpublished observations). Both FcεRI ligation and TSLP administration upregulated

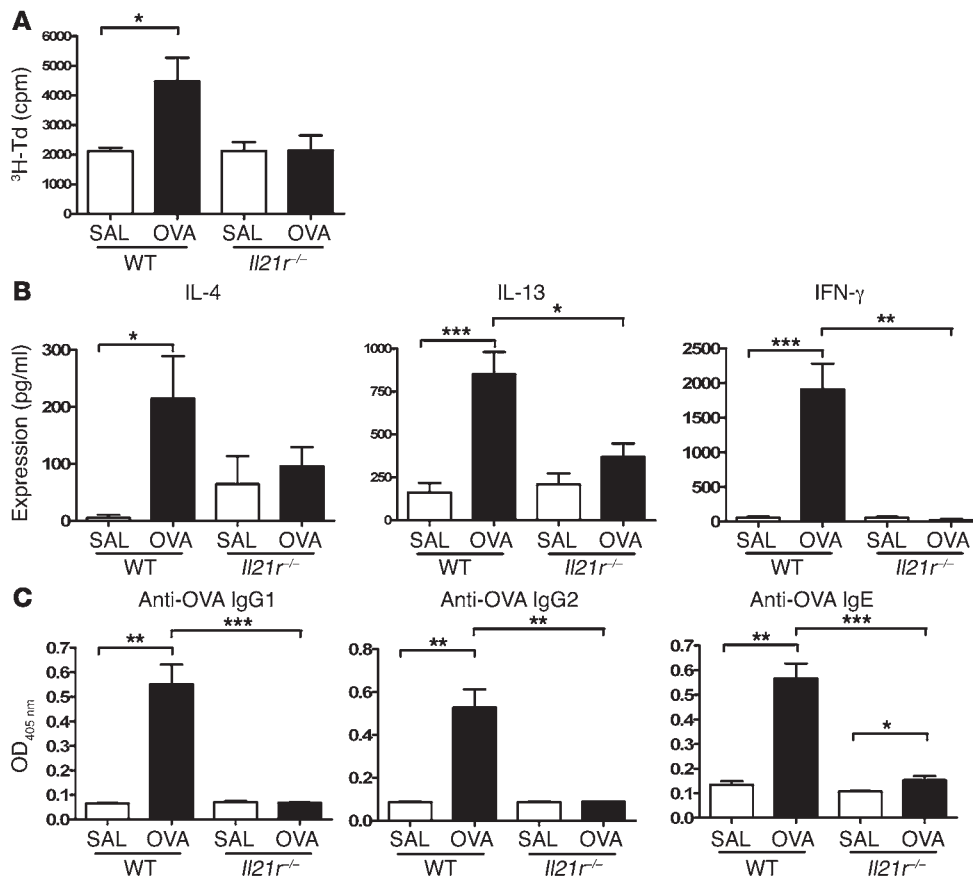
*Il21* mRNA expression in BMMCs (Figure 2E). These results suggest that both T cells and mast cells may contribute to increased expression of *Il21* mRNA in skin after tape stripping.

*IL-21R is required for allergic skin inflammation and for the systemic response induced by e.c. sensitization with OVA.* Tape stripping of the skin is critical for successful e.c. sensitization with protein antigen, because OVA sensitization of shaved, unstripped skin of WT mice and skin of hairless mice did not result in a systemic response to OVA (data not shown). To determine whether IL-21R is important for e.c. sensitization, we examined the response of *Il21r*<sup>-/-</sup> mice to e.c. application of OVA on tape-stripped skin. As was shown previously (24), OVA sensitization of the skin in WT Balb/c mice led to epidermal and dermal thickening as well as marked infiltration of the dermis with CD4<sup>+</sup> T cells and eosinophils (Figure 3, A and B). In contrast, there was no detectable epidermal or dermal thickening and weak infiltration of CD4<sup>+</sup> T cells and eosinophils in the dermis of OVA-sensitized skin of *Il21r*<sup>-/-</sup> mice. Expression of mRNA for the chemokine CCL17/



**Figure 3**

Impaired allergic skin inflammation and systemic response to e.c. sensitization in *Il21r<sup>-/-</sup>* mice. **(A)** H&E staining of sections from saline- (SAL) and OVA-sensitized skin. Higher magnification of the boxed regions showed the presence of multiple eosinophils (arrowheads) in OVA-sensitized skin of WT mice, but not *Il21r<sup>-/-</sup>* mice. Original magnification,  $\times 200$ ;  $\times 400$  (higher-magnification panels). **(B)** Cell counts for skin-infiltrating CD4<sup>+</sup> T cells and eosinophils. HPF, high-power field. **(C and D)** Quantitative RT-PCR analysis of mRNA levels of CCL17/TARC and CCL11/eotaxin-1 **(C)** and of IL-4, IL-13, and IFN- $\gamma$  **(D)**, expressed as fold induction compared with saline-sensitized skin sites. Values are mean  $\pm$  SD. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .  $n = 7$  per group.

**Figure 4**

Impaired systemic response to e.c. sensitization. (A and B) Proliferation (A) and cytokine secretion (B) by splenocytes in response to OVA stimulation *in vitro*.  $^3\text{H-Td}$ , [ $^3\text{H}$ ]-thymidine. (C) Serum levels of OVA-specific Ig isotypes. Values are mean  $\pm$  SD. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .  $n = 7$  per group.

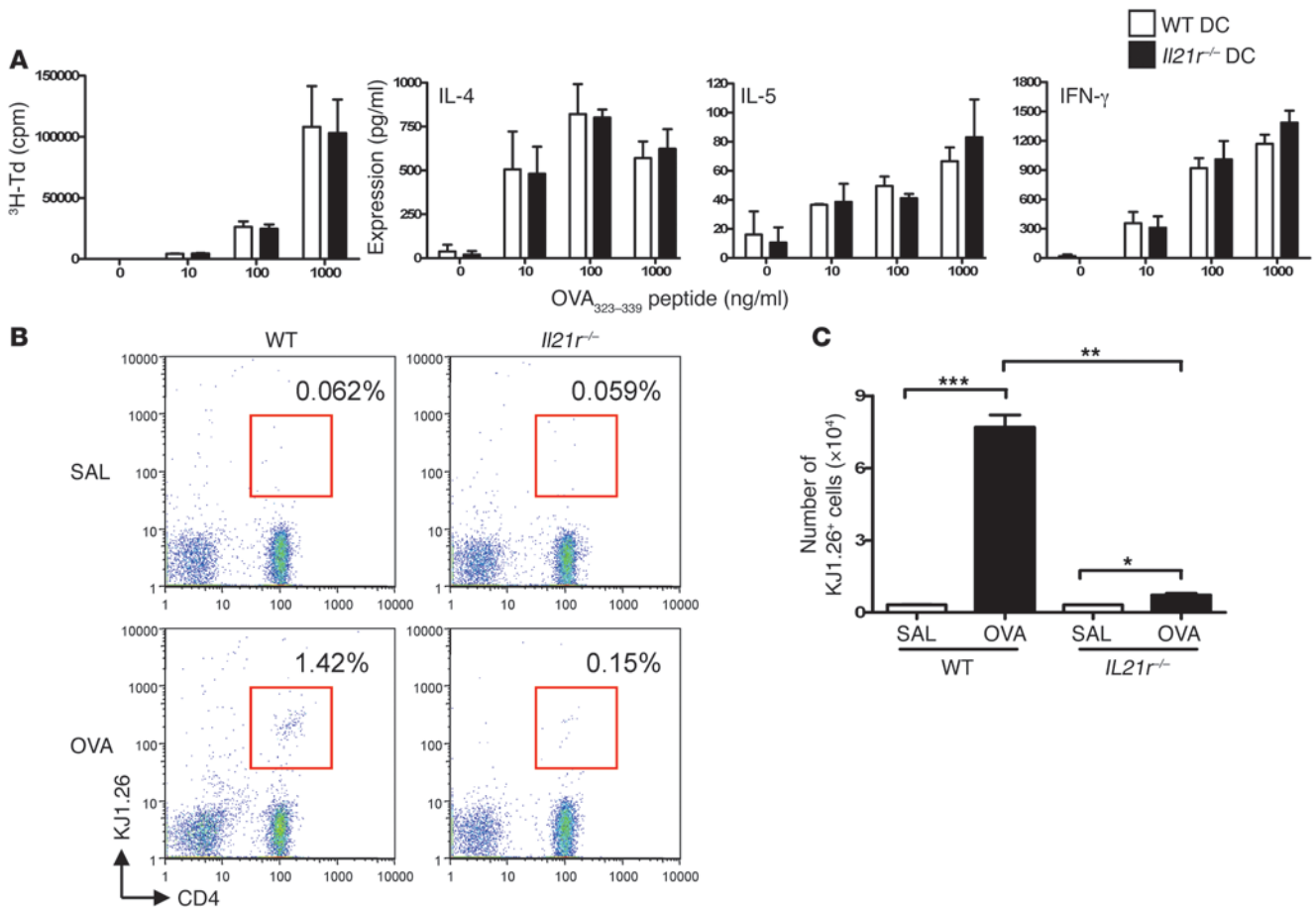
TARC, a ligand for CCR4, which is expressed on skin homing T cells, was significantly upregulated in OVA-sensitized skin from WT mice, but not  $Il21r^{-/-}$  mice (Figure 3C). OVA sensitization led to a significant upregulation of CCL11/eotaxin-1 mRNA in skin of WT mice, but not  $Il21r^{-/-}$  mice (Figure 3C). As we previously reported (24, 26), e.c. sensitization with OVA caused upregulation of IL-4 and IL-13, but not IFN- $\gamma$ , mRNA in the skin of WT Balb/c mice (Figure 3D). In contrast, there was no detectable upregulation of IL-4, IL-13, or IFN- $\gamma$  mRNA in OVA-sensitized skin of  $Il21r^{-/-}$  mice. These results demonstrate that IL-21R is essential for the development of allergic skin inflammation induced by e.c. introduced allergen.  $Il21r^{-/-}$  mice mounted a significantly diminished cutaneous hypersensitivity response to skin painting with the hapten oxazolone, as evidenced by significantly decreased ear swelling in response to ear skin challenge with oxazolone 24 and 72 h after challenge (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI32310DS1), which suggests that  $Il21r^{-/-}$  mice also have a defective response to cutaneously applied haptens.

**Defective systemic response to e.c. sensitization in  $Il21r^{-/-}$  mice.** We assessed the systemic immune response to e.c. introduced OVA. Splenocytes from e.c. sensitized WT mice proliferated and secreted IL-4, IL-13, and IFN- $\gamma$  in response to *in vitro* stimulation with OVA (Figure 4, A and B). In contrast, splenocytes from OVA-sensitized  $Il21r^{-/-}$  mice failed to proliferate and to secrete cytokines in response to OVA stimulation. WT Balb/c mice developed OVA-specific IgG1, IgG2a, and IgE antibodies following e.c. sensitization. In contrast,  $Il21r^{-/-}$  mice mounted virtually no OVA-specific IgG1 and IgG2a antibody responses to e.c. sensitization, and their

IgE anti-OVA response was severely diminished (Figure 4C). These results suggest that the absence of IL-21R impairs the development of a systemic response to e.c. sensitization.

*Il21r<sup>-/-</sup> DCs function normally in vitro, in contrast to impaired accumulation of adoptively transferred OVA-TCR transgenic WT T cells in DLNs of Il21r<sup>-/-</sup> mice.* Splenic DCs from  $Il21r^{-/-}$  mice are known to support T cell activation and proliferation normally (9). However, to our knowledge, the ability of DCs from skin DLNs of these mice to stimulate T cell proliferation and cytokine secretion has not yet been investigated.  $Il21r^{-/-}$  mice displayed no differences in DC subset composition in the steady state in ear skin, DLNs, and spleens, as determined by analysis of CD11c<sup>+</sup> populations for MHC class II, CD11b, Langerin, CD19, and B220 markers (Supplemental Table 1 and data not shown). Furthermore, the percentage of CCR7<sup>+</sup>CD11c<sup>+</sup> DCs in skin DLNs was comparable in WT and  $Il21r^{-/-}$  mice (Supplemental Figure 2), which suggests that the previously demonstrated baseline trafficking of skin DCs to DLNs (27) is independent of IL-21R signaling. Figure 5A shows that DCs from skin DLNs of  $Il21r^{-/-}$  and WT mice were comparable in their ability to stimulate proliferation and secretion of Th2 and Th1 cytokines by DO11.10 WT T cells in the presence of OVA peptide. Similar data were obtained with OVA protein (data not shown). These results indicate that DCs from skin DLNs of  $Il21r^{-/-}$  mice are intrinsically normal in their ability to process antigen and activate T cells.

Initiation of an immune response involves capture of antigen by tissue DCs and their subsequent activation and migration to DLNs, where they present antigenic peptides to circulating antigen-specific naive CD4<sup>+</sup> T cells, which then proliferate locally



**Figure 5**

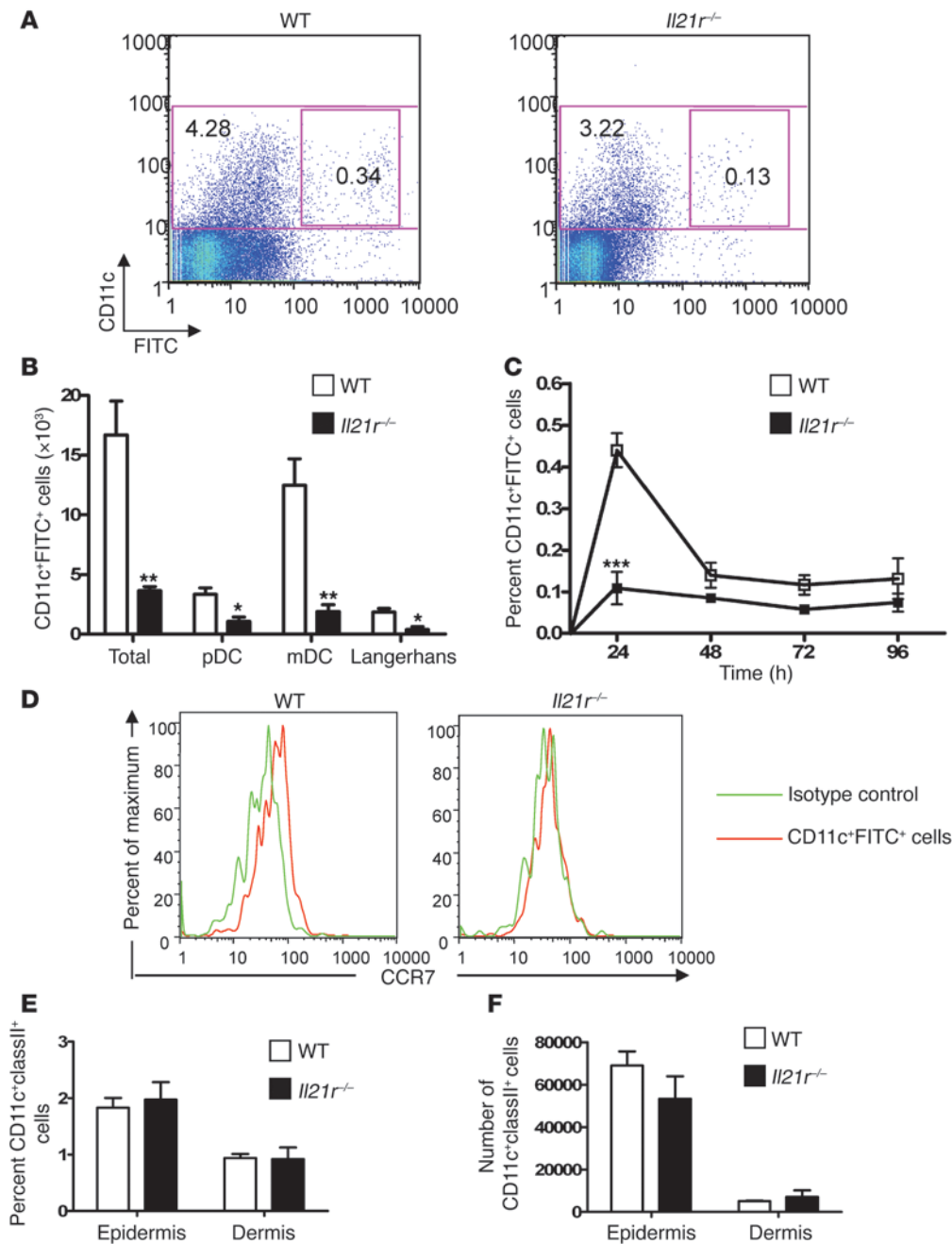
*Il21r<sup>-/-</sup>* DCs function normally in vitro, in contrast to impaired accumulation of adoptively transferred OVA-TCR transgenic WT T cells in DLNs of *Il21r<sup>-/-</sup>* mice. (A) Proliferation and cytokine secretion of DO11.10 WT CD4<sup>+</sup> T cells in response to OVA peptide presented by DCs from peripheral LNs of WT and *Il21r<sup>-/-</sup>* mice. (B and C) Analysis of CD4<sup>+</sup>KJ1.26<sup>+</sup> cells in the DLNs of recipients of CD4<sup>+</sup> OVA-TCR transgenic WT cells. Shown are representative FACS plots (B) and pooled results of 3 experiments (C). Percentages within plots denote percent cells within the boxed regions. \**P* < 0.05; \*\**P* < 0.01; \*\*\**P* < 0.001.

before reentering the circulation (28). To examine whether the defective response of *Il21r<sup>-/-</sup>* mice to e.c. sensitization involved defective interaction between DCs and T cells in vivo, we examined the ability of *Il21r<sup>-/-</sup>* mice to support the expansion of adoptively transferred WT TCR-OVA transgenic T cells in DLNs of OVA-sensitized skin. Naive CD4<sup>+</sup> DO11.10 WT cells were adoptively transferred into WT or *Il21r<sup>-/-</sup>* recipients, immediately followed by e.c. sensitization with OVA or saline on the tape-stripped skin. Skin DLNs were analyzed 4 days later for accumulation of CD4<sup>+</sup>KJ1.26<sup>+</sup> cells. Figure 5, B and C, show that both the percentage and the total number of CD4<sup>+</sup>KJ1.26<sup>+</sup> cells increased in DLNs of OVA-challenged skin compared with DLNs of saline-challenged skin in WT recipients. This accumulation was associated with cell division, as demonstrated using CFSE-loaded cells (data not shown). Accumulation of CD4<sup>+</sup>KJ1.26<sup>+</sup> cells was drastically reduced in DLNs of OVA-challenged skin in *Il21r<sup>-/-</sup>* recipients. As previously reported (7), the total number of CD4<sup>+</sup> cells and DCs in *Il21r<sup>-/-</sup>* DLNs was comparable to that in WT DLNs (data not shown).

*Impaired trafficking of skin DCs to DLNs.* A possible explanation for the defective response of *Il21r<sup>-/-</sup>* mice to e.c. sensitization is that skin DCs from these mice could be impaired in their ability to migrate to

DLNs after antigen capture. To directly test this hypothesis, we painted the shaved dorsal skin of mice with the hapten FITC, and 24 h later examined inguinal DLNs for the presence of CD11c<sup>+</sup>FITC<sup>+</sup> cells, which likely represent recent skin DC emigrants. Figure 6, A and B, show that the percentage and number of CD11c<sup>+</sup>FITC<sup>+</sup> DCs in DLNs of *Il21r<sup>-/-</sup>* mice was considerably reduced compared with WT DLNs. Defective trafficking affected all 3 subsets of DCs examined, which included myeloid DCs (CD11c<sup>hi</sup>CD11b<sup>+</sup>B220<sup>-</sup>CD19<sup>-</sup>Langerin<sup>-</sup>), plasmacytoid DCs (CD11c<sup>lo</sup>CD11b<sup>-</sup>B220<sup>+</sup>CD19<sup>-</sup>Langerin<sup>-</sup>), and Langerhans cells (CD11c<sup>+</sup>CD11b<sup>-</sup>B220<sup>-</sup>CD19<sup>-</sup>Langerin<sup>+</sup>; Figure 6B). The defective migration of skin DCs in *Il21r<sup>-/-</sup>* mice was not caused by delayed migration of these cells, as the percentage and number of CD11c<sup>+</sup>FITC<sup>+</sup> DCs in DLNs of *Il21r<sup>-/-</sup>* mice did not increase in the 24- to 96-h interval subsequent to FITC painting (Figure 6C). Furthermore, CD11c<sup>+</sup>FITC<sup>+</sup> DCs in DLNs of WT Balb/c mice, but not *Il21r<sup>-/-</sup>* mice, expressed CCR7 (Figure 6D).

A similar defect in the migration of CD11c<sup>+</sup>FITC<sup>+</sup> DCs to DLNs was observed when FITC was painted on shaved and tape-stripped skin (Supplemental Figure 3A). CD11c<sup>+</sup>FITC<sup>-</sup> DCs in these DLNs had a higher background MFI than did CD11c<sup>+</sup>FITC<sup>-</sup> DCs from DLNs of FITC-painted unstripped skin, possibly because of

**Figure 6**

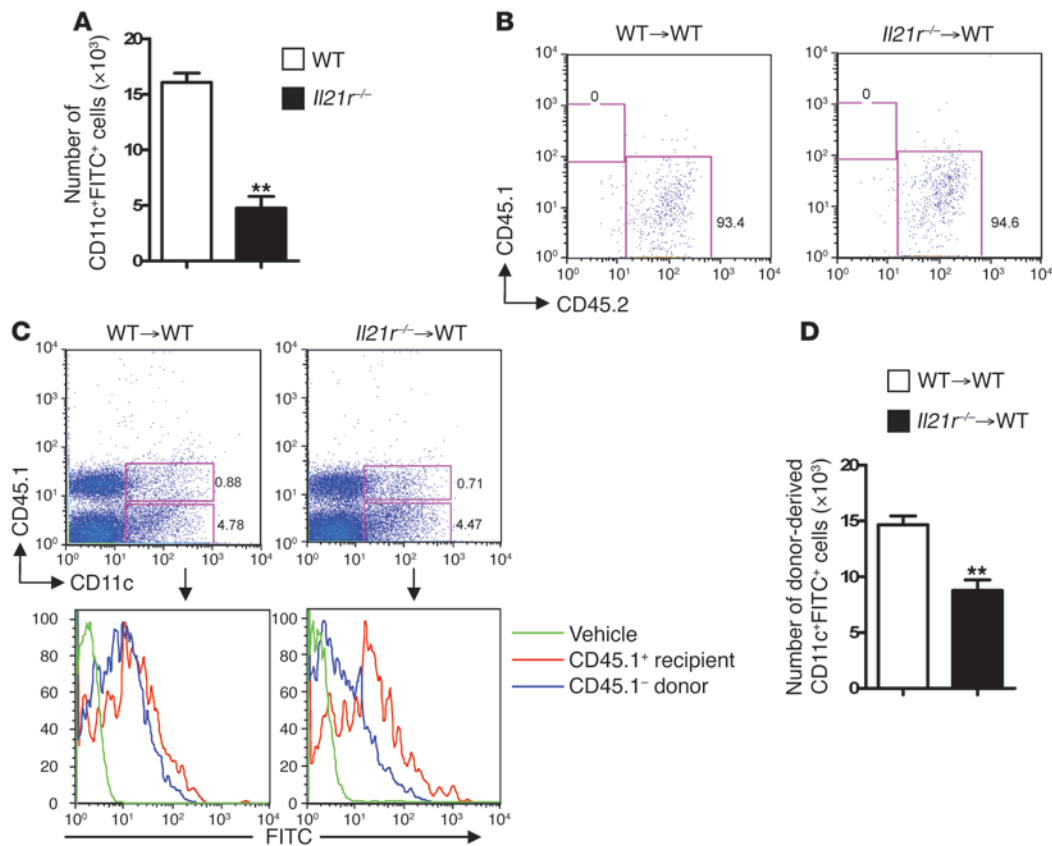
Defective trafficking of skin DCs in *Il21r*<sup>-/-</sup> mice. (A) Representative dot plots of FITC fluorescence plotted against CD11c in viable cells from DLNs of WT Balb/c and *Il21r*<sup>-/-</sup> mice 24 h after painting dorsal skin with FITC. Numbers within plots denote percent cells within the respective gates. (B) Number of total CD11c<sup>+</sup>FITC<sup>+</sup> DCs and of subsets of CD11c<sup>+</sup>FITC<sup>+</sup> DCs in DLNs of WT Balb/c and *Il21r*<sup>-/-</sup> mice ( $n = 3$  per group). mDC, myeloid DC; pDC, plasmacytoid DC. (C) Percent of CD11c<sup>+</sup>FITC<sup>+</sup> DCs in DLNs of WT Balb/c and *Il21r*<sup>-/-</sup> mice at the indicated time points ( $n = 3$  per group). (D) CCR7 expression by CD11c<sup>+</sup>FITC<sup>+</sup> DCs in DLNs of shaved skin 24 h after FITC painting. (E and F) Percent and number of CD11c<sup>+</sup>MHC class II<sup>+</sup> cells in epidermis and dermis of ears from *Il21r*<sup>-/-</sup> and WT mice ( $n = 4$  per group). Values are mean  $\pm$  SD. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  versus WT.

greater access of FITC to lymphatic ducts of tape-stripped skin. Defective migration of DCs from stripped skin to DLNs was supported by the observation that there was significantly more DC retention in the tape-stripped skin of *Il21r*<sup>-/-</sup> than WT mice (Supplemental Figure 3B).

Decreased DC trafficking in *Il21r*<sup>-/-</sup> mice was not caused by a reduction in the number of DCs in the skin, as the percentage and absolute number of CD11c<sup>+</sup>MHC class II<sup>+</sup> cells in ear epidermis and dermis were comparable in *Il21r*<sup>-/-</sup> and WT mice (Figure 6, E and F). Surface expression of CD83, CD86, MHC class II, and CD40 was upregulated on CD11c<sup>+</sup>FITC<sup>+</sup> DCs compared with CD11c<sup>+</sup>FITC<sup>-</sup> DCs in DLNs of WT mice (data not shown). Expression of these activation markers was comparable in CD11c<sup>+</sup>FITC<sup>+</sup>

DCs from *Il21r*<sup>-/-</sup> mice and WT controls (Supplemental Figure 4). These results suggest that IL-21R plays an important role in the migration of antigen-presenting DCs from skin to DLNs, but not in their expression of activation markers.

*The defect in migration of skin DCs in Il21r-/- mice involves cell-autonomous and non-cell-autonomous components.* To determine whether the defect in DC trafficking was cell autonomous, we examined the trafficking of skin DCs in chimeras in which BM from CD45.2<sup>+</sup> WT or *Il21r*<sup>-/-</sup> donors was used to reconstitute lethally irradiated CD45.1<sup>+</sup> recipients, all on the C57BL/6 background. We first confirmed that trafficking of CD11c<sup>+</sup>FITC<sup>+</sup> DCs from skin to DLNs significantly decreased in C57BL/6 *Il21r*<sup>-/-</sup> mice, as in Balb/c *Il21r*<sup>-/-</sup> mice (Figure 7A). Analysis of spleens from BM chi-



**Figure 7** Defective trafficking of skin DCs in *Il21r*<sup>-/-</sup> mice and in *Il21r*<sup>-/-</sup>→WT BM chimeras. (A) Number of CD11c<sup>+</sup>FITC<sup>+</sup> DCs in DLNs of FITC-painted skin of C57BL/6 mice. (B and C) Representative FACS analysis of donor-derived (CD45.2<sup>+</sup>) and recipient-derived (CD45.1<sup>+</sup>) DCs in spleens (B) and skin DLNs of BM chimeras (C, top). Numbers within plots denote percent cells within the boxed regions. FACS analysis of FITC fluorescence by CD11c<sup>+</sup> cells of donor and recipient origin in DLNs of BM chimeras is also shown (C, bottom). (D) Number of donor-derived CD11c<sup>+</sup>FITC<sup>+</sup> DCs in DLNs of mouse chimeras (*n* = 3 per group). Values are mean ± SD. \*\**P* < 0.01 versus respective WT or WT→WT control.

meras 9 weeks after reconstitution revealed that greater than 95% of CD11c<sup>+</sup> cells (Figure 7B) as well as T cells and B cells (data not shown) were donor derived. Analysis of skin DLNs in WT recipients revealed that greater than 85% of DCs were donor-derived CD11c<sup>+</sup>CD45.1<sup>-</sup> cells (Figure 7C, top). This level of reconstitution of DCs in DLNs of BM chimeras is consistent with a recent report (29). The remaining recipient-derived cells were likely radio-resistant Langerhans cells (30).

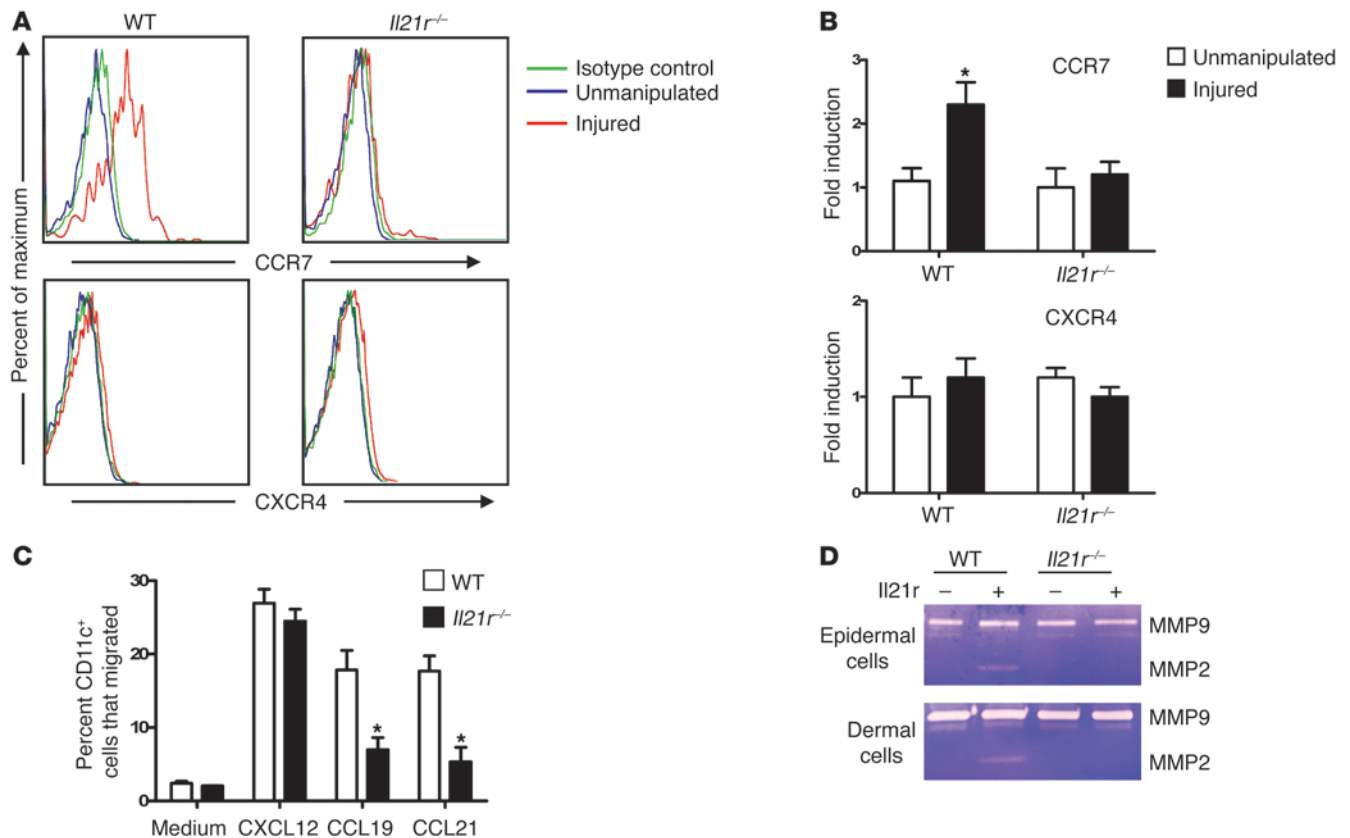
After FITC painting, the fraction of FITC<sup>+</sup> cells among CD11c<sup>+</sup> DCs of donor and recipient origin was comparable in DLNs of WT→WT chimeras (Figure 7C, bottom). In contrast, the fraction of FITC<sup>+</sup> cells among CD11c<sup>+</sup> DCs of donor (*Il21r*<sup>-/-</sup>) origin in *Il21r*<sup>-/-</sup>→WT chimeras was less than that found among DCs of recipient (WT) origin. Figure 7D shows that the number of donor-derived CD11c<sup>+</sup>FITC<sup>+</sup> DCs that accumulated in DLNs was significantly less in *Il21r*<sup>-/-</sup>→WT chimeras than in control WT→WT chimeras. These results suggest that *Il21r*<sup>-/-</sup> DCs have an intrinsic defect in their ability to traffic from skin to DLNs. However, the reduction in the number of CD11c<sup>+</sup>FITC<sup>+</sup> cells was less pronounced in the *Il21r*<sup>-/-</sup>→WT chimeras than in *Il21r*<sup>-/-</sup> mice (40.4% ± 6% versus 70.9% ± 9% reduction, *n* = 3; *P* < 0.05), which suggests contribution by nonhematopoietic cells to the DC trafficking defect in *Il21r*<sup>-/-</sup> mice. We were unable to examine DC trafficking in *Il21r*<sup>-/-</sup> C57BL/6 recipient chimeras in this study because *Il21r*<sup>-/-</sup> C57BL/6 mice have failed to breed in our facility.

*Impaired expression of CCR7 by skin DCs and impaired MMP2 activation in the epidermis following mechanical injury in Il21r-/- mice.* Mobilization of DCs requires expression of CCR7, the receptor for the chemokines CCL19 and CCL21, which are expressed

by high endothelial venules and stromal cells in LNs (27, 31). CD11c<sup>+</sup> cells from unmanipulated ear skin of *Il21r*<sup>-/-</sup> mice and WT controls had no detectable baseline surface expression of CCR7 and comparable baseline mRNA expression (Figure 8, A and B). Upregulation of CCR7 in skin CD11c<sup>+</sup> cells was observed 6–8 h after tape stripping in ears of WT but not *Il21r*<sup>-/-</sup> mice (Figure 8, A and B), but was no more evident at 24 h (data not shown), possibly because DCs that expressed CCR7 have migrated to DLNs. FITC<sup>+</sup> DCs in tape-stripped skin of *Il21r*<sup>-/-</sup> mice upregulated the activation markers CD83, CD86, MHC class II, and CD40 normally (Supplemental Figure 5A), which suggests that IL-21R signaling is not important for maturation of skin DCs. The influx of CD11b<sup>+</sup>Gr1<sup>+</sup> neutrophils into tape-stripped skin was comparable in *Il21r*<sup>-/-</sup> mice and WT controls (Supplemental Figure 5B), while influx of CD4<sup>+</sup> cells was undetectable in both strains (data not shown). Thus activated DCs that failed to emigrate from the skin of *Il21r*<sup>-/-</sup> mice did not result in increased influx of neutrophils or CD4<sup>+</sup> T cells.

Consistent with failure to upregulate CCR7, *in vitro* migration of CD11c<sup>+</sup> cells isolated from skin 6–8 h after tape stripping of ear skin toward CCL19 and CCL21 was significantly reduced in *Il21r*<sup>-/-</sup> mice (Figure 8C). Expression of CXCR4 and migration toward CXCL12 by DCs from tape-stripped ear skin were comparable in *Il21r*<sup>-/-</sup> and WT mice (Figure 8, A–C). Incubation with IL-21 for 24 h failed to cause detectable upregulation of CCR7 expression on CD11c<sup>+</sup> cells isolated from ear skin or on BM-derived DCs (BMDCs; data not shown). The biologic activity of the IL-21 preparation was validated by demonstrating its proapoptotic effect and CD23-downregulatory effect on LPS-



**Figure 8**

IL-21 mediates upregulation CCR7 expression in tape-stripped skin and induces activation of MMP2 in the epidermis and dermis. (A and B) FACS analysis of surface expression (A) and quantitative RT-PCR analysis of mRNA expression (B) of CCR7 and CXCR4 by ear skin CD11c<sup>+</sup> cells isolated from skin 6–8 h after tape stripping. (C) In vitro chemotaxis of CD11c<sup>+</sup>MHC class II<sup>+</sup> cells isolated from tape-stripped ear skin toward CXCL12, CCL19, and CCL21 ( $n = 3$  per group). (D) Gelatin zymography assay of MMP2 and MMP9 activity in supernatants of epidermal and dermal cells from WT and *Il21r*<sup>-/-</sup> mice. Results shown are representative of 2 experiments. Values are mean  $\pm$  SD. \* $P < 0.05$  versus respective unmanipulated or WT control.

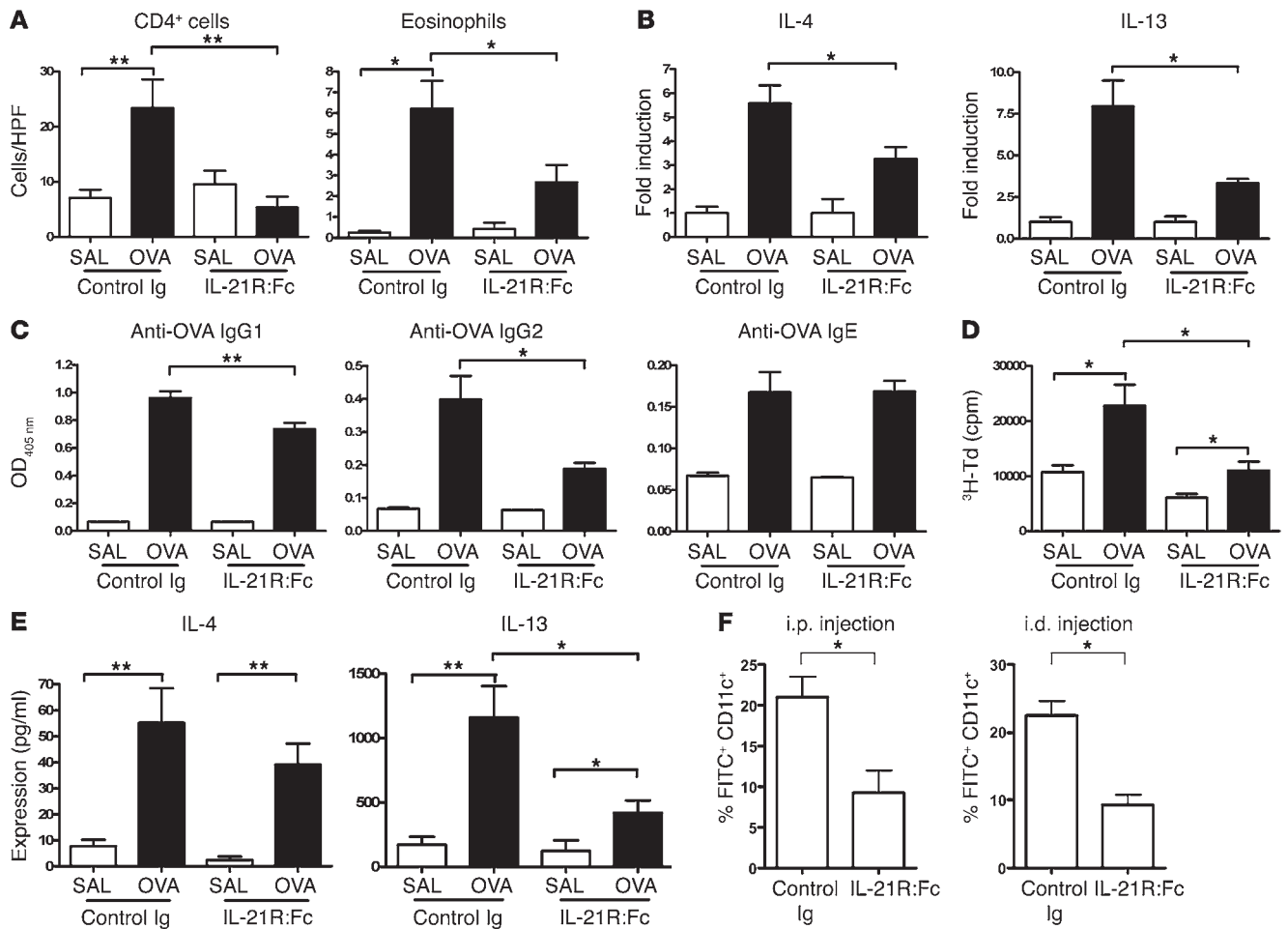
activated B cells (data not shown). These results suggest that mechanical injury by tape stripping upregulates CCR7 expression on skin DCs in an IL-21R-dependent, but indirect, manner that is likely to involve other skin cells.

MMP2 and MMP9 are expressed by skin Langerhans cells, dermal DCs, keratinocytes, and fibroblasts (32). Furthermore, migration of DCs from skin to regional LNs is dependent on the activation of MMP2 and MMP9 (33, 34). IL-21 upregulates the secretion of MMPs by human fibroblasts without affecting gene transcription or protein synthesis (35). We did not detect mRNA or protein changes of MMP2 or MMP9 by IL-21 in single-cell suspensions of epidermis and dermis. However, IL-21 activated the enzymatic activity of MMP2, but not MMP9, in single-cell suspensions from epidermis and dermis of WT mice, but not *Il21r*<sup>-/-</sup> mice (Figure 8D). IL-21 had no detectable effect on the expression or activation of MMP2 by BMDCs (data not shown).

*Administration of soluble IL-21R-IgG2aFc fusion protein inhibits the response to e.c. sensitization and DC trafficking.* Lack of IL-21R could have unknown effects on the development and maturation of immune cells in *Il21r*<sup>-/-</sup> mice. We therefore examined the effect of soluble IL-21R-IgG2aFc fusion protein (IL-21R:Fc) on the development of allergic inflammation. IL-21R:Fc was previously shown to effectively block IL-21 function in vivo (8, 36). Mice

received IL-21R:Fc or irrelevant IgG2a mAb i.p. 3 times per week throughout the 7-week period of e.c. sensitization. Allergic skin inflammation was significantly reduced in OVA-sensitized skin of mice that received IL-21R:Fc, but not in mice that received control IgG2a, as evidenced by a significant decrease in the number of infiltrating CD4<sup>+</sup> T cells and eosinophils (Figure 9A) and in the expression of the Th2 cytokines IL-4 and IL-13 (Figure 9B). Treatment with IL-21R:Fc inhibited the systemic response to e.c. sensitization, as evidenced by significantly decreased IgG1 and IgG2a, but not IgE, antibody responses to OVA compared with control-treated mice (Figure 9C). Splenocytes from IL-21R:Fc-treated mice proliferated and secreted IL-4, IL-13, and IFN- $\gamma$  in response to in vitro OVA restimulation significantly less than did splenocytes from control-treated mice (Figure 9, D and E, and data not shown).

We also examined the effect of IL-21 signaling blockade on the trafficking of skin DCs. Administration of IL-21R:Fc by the i.p. route or by local intradermal (i.d.) injection 24 h before FITC painting significantly inhibited the migration of CD11c<sup>+</sup>FITC<sup>+</sup> DCs to skin DLNs compared with administration of control IgG2a (Figure 9F). This result suggests that inhibition of e.c. sensitization by IL-21R:Fc involved disruption of trafficking of skin DCs, as we observed in *Il21r*<sup>-/-</sup> mice.



**Figure 9** IL-21R:Fc inhibits allergic inflammation and migration of skin DCs in e.c. sensitized WT mice. (A and B) Skin infiltration of CD4<sup>+</sup> T cells and eosinophils (A) and expression of mRNA levels for IL-4 and IL-13 (B) in WT Balb/c mice treated with IL-21R:Fc or control IgG2a. (C) Serum levels of OVA-specific Ig isotopes. (D and E) Proliferation (D) and cytokine secretion (E) by splenocytes in response to OVA stimulation in vitro. (F) Effect of i.p. and local i.d. administration of IL-21R:Fc on migration of skin DCs to DLNs. Values are mean ± SD. n = 6 per group. \*P < 0.05, \*\*P < 0.01.

**Discussion**

Our study demonstrates that absence of IL-21R results in defective migration of antigen-presenting DCs from skin to DLNs, defective immune response to antigen introduced e.c., and reduced skin inflammation. IL-21 and IL-21R protein expression was not detectable or negligible in normal human skin. In contrast, IL-21 protein was strongly expressed in mononuclear cells that infiltrate the dermis in skin lesions from AD patients and was also associated with keratinocytes, possibly because it was bound to its receptor on these cells. IL-21R protein was strongly expressed by keratinocytes in AD lesions (Figure 1). These observations prompted us to examine the potential role of IL-21R in the pathogenesis of allergic skin inflammation in a mouse model that is dependent on e.c. sensitization with antigen application to skin mechanically injured by tape stripping. Both tape stripping (mechanical injury) and application of acetone/dibutylphthalate (chemical injury) modestly upregulated *Il21* mRNA expression and strongly upregulated *Il21r* mRNA expression in mouse skin (Figure 2, A and B). We showed that, in addition to CD4<sup>+</sup> cells, mast cells are a potential source of IL-21 in skin, and that the keratinocyte-derived cytokine TSLP

upregulated IL-21 expression in mast cells (Figure 2E). Increased expression of IL-6 and TSLP after skin injury may upregulate IL-21 expression in skin CD4<sup>+</sup> cells and mast cells, respectively. *Il21r* mRNA is expressed by keratinocytes, T cells, BMDCs, and mast cells (refs. 37–39 and our unpublished observations). One or more of these cell lineages may account for increased expression of *Il21r* mRNA after skin injury.

*Il21r*<sup>-/-</sup> mice were severely deficient in their ability to develop allergic skin inflammation following e.c. sensitization. Dermal infiltration with CD4<sup>+</sup> T cells and eosinophils and Th2 cytokine expression in OVA-sensitized skin sites severely decreased in these mice (Figure 3, A, B, and D). In addition, upregulation of *Il17* mRNA, which we recently described in e.c. sensitized skin of WT mice (40), was not observed in *Il21r*<sup>-/-</sup> mice (data not shown). *Il21r*<sup>-/-</sup> mice also failed to upregulate local expression of TARC and eotaxin-1 (Figure 3C), key chemokines involved in homing to skin of T cells and eosinophils, respectively, whose expression is induced by IL-4 and IL-13 (25, 41–43). The impaired response of *Il21r*<sup>-/-</sup> mice to cutaneous sensitization was not restricted to OVA. *Il21r*<sup>-/-</sup> mice had a significantly diminished cutaneous hypersensitivity response to



the hapten oxazolone (Supplemental Figure 1), which suggests that IL-21R is important for the response to antigens other than OVA introduced through the skin.

The failure of *Il21r*<sup>-/-</sup> mice to develop allergic skin inflammation was likely caused by their severely impaired systemic response to e.c. sensitization. Splenocytes from *Il21r*<sup>-/-</sup> mice failed to proliferate and secrete both Th2 and Th1 cytokines in response to OVA stimulation in vitro (Figure 4, A and B). This contrasts with the reported normal secretion of Th2 cytokines and elevated secretion of IFN- $\gamma$  by splenocytes from these mice after i.v. or s.c. infection with parasites that migrate to tissues and after i.p. immunization with soluble antigen (8, 9). This suggests that the impaired Th response of *Il21r*<sup>-/-</sup> mice may be selective to the e.c. sensitization route. Consistent with the impaired systemic Th response to e.c. sensitization, OVA-specific IgG1, IgG2a, and IgE antibody responses were severely impaired in *Il21r*<sup>-/-</sup> mice (Figure 4C), and these mice failed to develop airway inflammation and methacholine hypersensitivity in response to inhalation challenge with OVA (Supplemental Figure 6). It has previously been reported that IL-21 suppresses Treg function (44). We found that the percentage of CD4<sup>+</sup>Foxp3<sup>+</sup> Tregs slightly decreased in the spleens of *Il21r*<sup>-/-</sup> mice (5.9%  $\pm$  0.6%,  $n = 4$ ) compared with WT controls (7.3%  $\pm$  0.5%,  $n = 5$ ) and that the ability of CD4<sup>+</sup>CD25<sup>+</sup> cells to suppress the proliferation of CD4<sup>+</sup>CD25<sup>-</sup> cells was intact in *Il21r*<sup>-/-</sup> mice (data not shown). Thus, it is unlikely that excessive activity of Tregs contributed to the impaired T cell response of *Il21r*<sup>-/-</sup> mice to e.c. immunization.

*Il21r*<sup>-/-</sup> T cells that bear a transgenic TCR for SMARTA2 proliferate normally in response to antigen in vitro (9). We found that *Il21r*<sup>-/-</sup> DCs supported normal proliferation and cytokine secretion by TCR transgenic WT T cells (Figure 5A), which indicates that IL-21R signaling is not important for DC/T cell interaction in vitro. However, DC/T cell interaction in vivo after e.c. sensitization was deficient in *Il21r*<sup>-/-</sup> mice, as evidenced by impaired accumulation of adoptively transferred TCR-OVA transgenic T cells in DLNs of OVA-sensitized skin in these mice (Figure 5, B and C). We formally demonstrated a defect in the trafficking of skin DCs to DLNs, as evidenced by significantly impaired accumulation of CD11c<sup>+</sup>FITC<sup>+</sup> cells in DLNs of *Il21r*<sup>-/-</sup> mice after FITC painting of skin (Figure 6, A–C, and Supplemental Figure 2A). CD11c<sup>+</sup>FITC<sup>+</sup> cells in DLNs likely represent skin DC emigrants rather than LN resident DCs that picked up FITC conveyed through lymphatic conduits (45). CD11c<sup>+</sup>FITC<sup>+</sup> cells in DLNs in *Il21r*<sup>-/-</sup> mice were too few to allow direct examination of their antigen-presenting function; thus we cannot formally rule out a role for IL-21/IL-21R interactions in the polarization of skin DCs. IL-21 has previously been reported to inhibit the ability of BMDCs to activate T cells in vitro and to elicit contact hypersensitivity when injected into WT mice (46). It has also been reported that *Il21r*<sup>-/-</sup> mice immunized s.c. with trinitrophenyl hapten–keyhole limpet hemocyanin (TNP-KLH), then challenged on the footpad 6 days later, exhibit increased footpad swelling and IFN- $\gamma$  production in DLNs (47). These 2 reports suggest that IL-21 may inhibit contact hypersensitivity, but neither of them examined DC migration from skin. Injection of mouse recombinant IL-21 i.d. in the skin of WT mice at the site of FITC application did not result in increased migration of FITC<sup>+</sup> DCs to DLNs (Supplemental Figure 7), which suggests that sufficient IL-21 is released in WT skin for optimal DC migration.

The defect in the trafficking of skin DCs to DLNs was cell intrinsic, at least in part, because the number of donor-derived CD11c<sup>+</sup>FITC<sup>+</sup> cells that accumulated in DLNs was less in *Il21r*<sup>-/-</sup> $\rightarrow$ WT than in con-

trol WT $\rightarrow$ WT BM chimeras (Figure 7D). However, the defect in DC trafficking was less pronounced in the *Il21r*<sup>-/-</sup> $\rightarrow$ WT chimeras than in *Il21r*<sup>-/-</sup> mice (Figure 7, A and D), which suggests a contribution by nonhematopoietic cells to the trafficking defect of skin DCs in *Il21r*<sup>-/-</sup> mice or a bystander effect of residual DCs of recipient origin.

Migration of skin DCs to DLNs is regulated by the interaction of the chemokines CCL19 and CCL21 with their receptor, CCR7 (27, 31). Expression of CCR7 by skin CD11c<sup>+</sup> DCs was upregulated by mechanical injury in WT mice (Figure 8A). This upregulation was severely impaired in *Il21r*<sup>-/-</sup> mice and was associated with impaired ability of DCs to migrate toward CCR7 ligands (Figure 8, A–C). Furthermore, CD11c<sup>+</sup>FITC<sup>+</sup> cells isolated from DLNs of shaved skin painted with FITC had impaired CCR7 expression (Figure 6D). STAT3 is activated by IL-21 (48), and the CCR7 gene contains a conserved STAT-binding consensus sequence (TTCN<sub>2-4</sub>GAA) in its 5' upstream region. However, although *Il21r* mRNA is expressed in BMDCs (39), we were unable to detect IL-21R surface expression on DCs isolated from mouse skin by FACS or to detect upregulation of CCR7 on these DCs or on BMDCs by IL-21 (data not shown). In light of its critical role in CCR7 expression by skin DCs in vivo, the failure of IL-21 to induce CCR7 on BMDCs in vitro suggests that IL-21R is necessary, but not sufficient, for CCR7 upregulation by skin DCs in injured skin. IL-21 could target one or more non-DC skin cells, which may in turn induce CCR7 expression on skin DCs. Alternatively, signaling by surface IL-21R expressed below the FACS detection limit could synergize with an additional signal to upregulate CCR7 expression on DCs, as suggested by the observation that IL-21 synergizes with TCR signaling to cause upregulation of CCR7 on CD8<sup>+</sup> cells (49). The latter scenario is consistent with both DC-intrinsic and nonintrinsic components to the DC trafficking defect.

Migration of DCs from skin to DLNs is dependent on the activation of MMPs (32). IL-21 activated MMP2, but not MMP9, in cells from epidermis and dermis of WT mice, but not *Il21r*<sup>-/-</sup> mice (Figure 8D). Lack of MMP2 activation in the skin by IL-21 may contribute to defective trafficking of DCs to DLNs in *Il21r*<sup>-/-</sup> mice. IL-21 caused no detectable activation of MMP2 or upregulation of its level in BMDCs, suggesting the involvement of cells other than DCs in IL-21-driven MMP2 activation in the skin.

The defects in the immune response of *Il21r*<sup>-/-</sup> mice to e.c. sensitization were reproduced in WT mice treated with IL-21R:Fc (Figure 9, A–E). More importantly, DC migration from skin to regional LNs was significantly impaired in WT mice treated with IL-21R:Fc (Figure 9F). These results rule out developmental effects as an explanation for the impaired response of *Il21r*<sup>-/-</sup> mice to e.c. sensitization and for the impaired trafficking of skin DCs in these mice.

AD is initiated and perpetuated by recurrent mechanical injury inflicted by scratching and introduction of antigens via the skin (50). We showed here that skin injury upregulated *Il21r* mRNA expression, and to a lesser extent *Il21* mRNA expression, in skin and that IL-21R was critical for the upregulation of CCR7 expression by skin DCs and for their migration to DLNs, where they initiated the immune response to e.c. introduced antigen that results in allergic skin inflammation. Increased expression of IL-21 and IL-21R in AD lesions as a result of infiltration by inflammatory cells and possibly scratching could further enhance the migration of DCs that carry antigens from lesional skin to DLNs. This may result in further and more efficient sensitization to environmental allergens that would exacerbate and perpetuate the disease. Blockade of IL-21/IL-21R interaction in the skin could be a useful strategy for the prevention of cutaneous sensitization in AD.



## Methods

**Expression of IL-21 and IL-21R protein in human skin.** Skin biopsies were obtained from normal donors undergoing surgery and from skin lesions of patients with AD at the University of Bonn after securing informed consent. Heat-induced antigen retrieval of multiple 4- $\mu$ m sections of human skin was performed in pH 9 heated retrieval solution for 10 min. The recovered sections were stained with polyclonal rat anti-human IL-21, polyclonal rat anti-human IL-21R, or rat Ig control (eBiosciences). The immunoreaction was revealed by a standard LSAB-alkaline phosphatase method (LSAB2; Dako) using fast red as chromogen. The slides were read blinded. Cutaneous expression of IL-21 and IL-21R was scored semiquantitatively (0, no expression; 1, weak expression; 2, moderate expression; 3, strong expression) as described previously (51). All skin samples were obtained after securing informed consent from individuals in accordance with the ethics committee from the medical center of the Friedrich-Wilhelms University of Bonn.

**Mice.** Balb/c and C57BL/6 WT mice and DO11.10 TCR transgenic mice were purchased from Charles River Laboratory. SJL-PtprcaPep3b/BoyJ (CD45.1 congenic) mice were purchased from Taconic Farms. *Il21r*<sup>-/-</sup> mice on Balb/c and C57BL/6 backgrounds were generated as reported previously (52, 53). All mice were kept in a pathogen-free environment and fed an OVA-free diet. All procedures performed on the mice were in accordance with the Animal Care and Use Committee of the Children's Hospital Boston.

**e.c. sensitization.** Female mice (4–6 weeks old) were sensitized e.c. as described previously (24). Briefly, the back skin of anesthetized mice was shaved and tape stripped 6 times. OVA (100  $\mu$ g, Grade V; Sigma-Aldrich) in 100  $\mu$ l normal saline or placebo (100  $\mu$ l normal saline) was placed on a patch of sterile gauze (1  $\times$  1 cm), which was secured to the skin with a transparent bio-occlusive dressing (Tegaderm; Westnet Inc.). Each mouse had a total of 3 1-week exposures to the patch separated by 2-week intervals. Mice were sacrificed immediately at the end of the third cycle of sensitization, on day 49.

**Histological and immunohistochemical analysis of mouse skin.** Skin specimens were fixed in 10% buffered formalin and embedded in paraffin. Multiple 4- $\mu$ m sections of skin were stained with H&E by Histo-Scientific Research Laboratories. CD4 staining of skin sections was performed as previously described (24). Eosinophils and CD4<sup>+</sup> cells were counted blinded in 10–15 high-power fields at  $\times$ 600 magnification.

**Derivation and activation of BMMCs.** BMMCs were obtained as previously described by culturing BM cells in WEHI-3-conditioned medium as a source of IL-3 (54). After 3–5 weeks of culture, more than 90% of the cells were mast cells, as evidenced by FACS analysis for IgE receptor using PE-conjugated anti-mouse Fc $\epsilon$ RI $\alpha$  (catalog no. 12-5898-82; eBiosciences). For activation of BMMCs, 1  $\times$  10<sup>6</sup> cells were incubated overnight with 0.25  $\mu$ g mouse IgE anti-DNP mAb (clone no. SPE7; Sigma-Aldrich). The next day, the cells were washed with medium, and IgE antibody was cross-linked with 30 ng/ml DNP-HSA (Sigma-Aldrich) for 8 h.

**Quantitative RT-PCR for cytokines, chemokines, and receptors.** Specimens of skin and lung 100 mg in weight were homogenized using a Polytron RT-3000 (Kinematica; AG) in lysis buffer solution provided in the RNAqueous Extraction Kit (Ambion Inc.). Reverse transcription was performed using iScript cDNA synthesis kit (BioRad). PCR reactions were run on an ABI Prism 7300 (Applied Biosystems) sequence detection system platform. Taqman primers and probes were obtained from Applied Biosystems. The housekeeping genes  $\beta$ <sub>2</sub>-microglobulin and GAPDH were used as controls. Relative gene expression was determined using the method described by Pfaffl (55).

**Il21 and Il21r mRNA expression in different cell types by RT-PCR.** BMDs and BMMCs were prepared according to previously described protocols (56, 57). PCR reactions were performed on cDNA using RT-PCR primer sequences for the following mouse genes: *Il21r*, 5'-GGACAGTG-

GCCCATAATCAAG-3' and 5'-AATTCTTTGGGTGTCCTTTTCTCA-3'; *Il21r*, 5'-AGTGCCCCAGCCTAAAGAAT-3' and 5'-ACTGAGTATGCTGGGGTTGG-3'; *Gapdh*, 5'-ATGACATCAAGAAGGTGGTG-3' and 5'-CATAACCAGGAATGAGCTTG-3'. The number of PCR cycles was 40 for *Il21* and 35 for *Il21r* and *Gapdh*.

**Serum antibody determination.** The BD Biosciences – Pharmingen protocol for sandwich ELISA was used to quantify serum total Igs. OVA-specific IgG1, IgG2a, and IgE antibodies were determined by ELISA as previously described (24).

**Cell culture, proliferation assay, and in vitro cytokine production.** Single-cell suspensions of spleen cells were cultured in complete RPMI 1640 (JRH Biosciences Inc.) supplemented by 10% FCS, 1 mM sodium pyruvate, 2 mM L-glutamine, 0.05 mM 2-mercaptoethanol, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin at 2  $\times$  10<sup>5</sup>/ml in 96-well plates or 2  $\times$  10<sup>6</sup>/ml in 24-well plates in the presence of 50  $\mu$ g/ml OVA. Proliferation was measured in triplicate wells of 96-well plates by [<sup>3</sup>H]-thymidine incorporation after 72 h of culture. Cytokine secretion in supernatants from 24-well plates after 96 h of culture was determined by ELISA according to the manufacturer's instructions (BD Biosciences – Pharmingen).

**In vivo assessment of antigen-specific T cell accumulation.** OVA-specific DO11.10 CD4<sup>+</sup> T cells sorted using mouse CD4<sup>+</sup> Miltenyi isolation kit were injected i.v. into WT Balb/c or *Il21r*<sup>-/-</sup> recipients that were immediately tape stripped and sensitized with 100  $\mu$ g OVA or saline on dorsal skin. In vivo accumulation of adoptively transferred cells in the inguinal LNs was analyzed by FACS staining using the anti-clonotypic mAb KJ1.26 (eBioscience).

**In vitro assessment of APC function of DCs.** CD11c<sup>+</sup> DCs were sorted from skin DLNs of WT or *Il21r*<sup>-/-</sup> mice using Miltenyi mouse CD11c beads and cocultured with WT DO11.10 CD4<sup>+</sup> T cells at a 1:10 ratio in the presence of graded concentrations (0, 10, 100, and 1,000 ng/ml) of OVA<sub>323–339</sub> peptide. After 96 h, T cell proliferation and cytokine production were measured as described above.

**In vivo assay for migration of skin DCs.** The dorsal skin of individual mice was shaved, or shaved and tape stripped, followed by application of 100  $\mu$ l of 10 mg/ml FITC dissolved in 1:1 acetone/dibutylphthalate (Sigma-Aldrich). At 24, 48, 72, and 96 h, inguinal LNs were isolated by digestion at 37°C for 1 h with a cocktail of 0.1% DNase I (fraction IX; Sigma-Aldrich) and 1.6 mg/ml collagenase (CLS4; Worthington Biochemical). Single-cell suspensions were incubated with Fc-block (Fc $\gamma$ RII/III mAb 2.4G2) for 15 min and stained with antibodies to CD11c, MHC class II, CD11b, CD19, and Langerin (all from eBioscience) as well as B220 (BD Biosciences). The stained cells were washed and analyzed on a FACSCanto flow cytometer (BD).

**BM reconstitution assay.** Red blood cell-free BM cells (1  $\times$  10<sup>6</sup>) from CD45.2<sup>+</sup> WT or *Il21r*<sup>-/-</sup> mice on C57BL/6 background were injected i.v. into lethally irradiated CD45.1 congenic SJL-PtprcaPep3b/BoyJ mice as previously described (58). Recipients were kept in autoclaved cages and fed with trimethoprim sulfamethoxazole-containing water for 9 weeks.

**Analysis of epidermal and dermal cells.** Preparation of dermal and epidermal sheets was performed as described previously (59). Unmanipulated ears or ears taken 6 or 12 h after tape stripping were floated on 0.25% trypsin and 2.2 mM EDTA for 25–45 min at 37°C, and epidermis was peeled off. After separation, epidermal and dermal sheets were further incubated in RPMI 1640 containing 10% FCS at 37°C for 1 and 2 h, respectively. Single-ear cell suspensions were stained with Abs for MHC class II, CD11c, and CCR7 (Serotec) as well as CXCR4 and IL-21R (BD Biosciences), followed by FACS analysis. Total ear cell suspensions were subjected to cell sorting for CD11c<sup>+</sup> population, which were analyzed for CCR7 and CXCR4 mRNA expression by real-time PCR.

**Chemotaxis assays.** Chemotaxis assays were performed as previously described (60). Single-cell suspension of total ear cells was placed into the



upper compartment ( $1 \times 10^6$  cells/well) of Transwell inserts (Costar). The lower compartment contained medium plus 200 ng/ml CXCL12, 250 ng/ml CCL19, or 250 ng/ml CCL21 (R&D Systems) in RPMI 1640 containing 0.25% BSA. Cells were allowed to migrate for 2 h at 37°C. Input cells and migrated cells were stained with Abs for MHC class II and CD11c. Cells were counted by timed acquisition (600 s per sample) on a FACSCanto flow cytometer (BD).

**MMP activity.** MMP activity was determined by gelatin zymography. Briefly, supernatants of epidermal cells incubated 24 h with or without IL-21 were electrophoresed on SDS-PAGE gel containing 0.1% gelatin. Gels were treated with 2.5% Triton-100 for 30 min at room temperature, followed by an incubation with zymogram developing buffer containing 50 mM Tris-HCl, 0.2 M NaCl, 5 mM CaCl<sub>2</sub>, and 0.02% Brij35 at 37°C. Finally, the gel was stained with 0.5% (w/v) Coomassie Blue R-250 for 30 min and destained in 50% methanol and 10% glacial acid.

**In vivo blockade of IL-21/IL-21R interaction.** WT Balb/c mice were injected i.p. with 180 µg IL-21R:Fc or control IgG2a mAb to *Eimeria tenella* 3 times per week throughout the 7-week period of e.c. sensitization. Other groups of WT Balb/c mice were injected i.p. or i.d. with 18 µg IL-21R:Fc or control IgG2a mAb 24 h before FITC painting of shaved and tape-stripped skin.

**Statistics.** Statistical analysis for human immunohistology was performed using SPSS software (version 14; SPSS Inc.). The nonparametrical Mann-Whitney *U* test was used to compare the expression of IL-21 and IL-21R in patients with AD and healthy controls. All other experiments were analyzed using the 2-tailed Student's *t*-sample *t* test or ANOVA with

Prism software (version 4; GraphPad). Results are expressed as mean ± SD. A *P* value less than 0.05 was considered significant.

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- Mehta, D.S., Wurster, A.L., and Grusby, M.J. 2004. Biology of IL-21 and the IL-21 receptor. *Immunol. Rev.* **202**:84–95.
- Coquet, J.M., et al. 2007. IL-21 is produced by NKT cells and modulates NKT cell activation and cytokine production. *J. Immunol.* **178**:2827–2834.
- Spolski, R., and Leonard, W.J. 2008. Interleukin-21: basic biology and implications for cancer and autoimmunity. *Annu. Rev. Immunol.* **26**:57–79.
- Suto, A., Wurster, A.L., Reiner, S.L., and Grusby, M.J. 2006. IL-21 inhibits IFN- $\gamma$  production in developing Th1 cells through the repression of Eomesodermin expression. *J. Immunol.* **177**:3721–3727.
- Strengell, M., et al. 2003. IL-21 in synergy with IL-15 or IL-18 enhances IFN- $\gamma$  production in human NK and T cells. *J. Immunol.* **170**:5464–5469.
- Zeng, R., et al. 2005. Synergy of IL-21 and IL-15 in regulating CD8<sup>+</sup> T cell expansion and function. *J. Exp. Med.* **201**:139–148.
- Ozaki, K., et al. 2002. A critical role for IL-21 in regulating immunoglobulin production. *Science*. **298**:1630–1634.
- Pesce, J., et al. 2006. The IL-21 receptor augments Th2 effector function and alternative macrophage activation. *J. Clin. Invest.* **116**:2044–2055.
- Frohlich, A., et al. 2007. IL-21 receptor signaling is integral to the development of Th2 effector responses in vivo. *Blood*. **109**:2023–2031.
- Nurieva, R., et al. 2007. Essential autocrine regulation by IL-21 in the generation of inflammatory T cells. *Nature*. **448**:480–483.
- Korn, T., et al. 2007. IL-21 initiates an alternative pathway to induce proinflammatory T(H)17 cells. *Nature*. **448**:484–487.
- Zhou, L., et al. 2007. IL-6 programs T(H)-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways. *Nat. Immunol.* **8**:967–974.
- Brady, J., Hayakawa, Y., Smyth, M.J., and Nutt, S.L. 2004. IL-21 induces the functional maturation of murine NK cells. *J. Immunol.* **172**:2048–2058.
- Roda, J.M., et al. 2006. Interleukin-21 enhances NK cell activation in response to antibody-coated targets. *J. Immunol.* **177**:120–129.
- Takaki, R., et al. 2005. IL-21 enhances tumor rejection through a NKG2D-dependent mechanism. *J. Immunol.* **175**:2167–2173.
- Ettinger, R., et al. 2007. IL-21 and BAFF/BLyS synergize in stimulating plasma cell differentiation from a unique population of human splenic memory B cells. *J. Immunol.* **178**:2872–2882.
- Erazo, A., et al. 2007. Unique maturation program of the IgE response in vivo. *Immunity*. **26**:191–203.
- Laughter, D., Istrvan, J.A., Tofte, S.J., and Hanifin, J.M. 2000. The prevalence of atopic dermatitis in Oregon schoolchildren. *J. Am. Acad. Dermatol.* **43**:649–655.
- Leung, D.Y. 2000. Atopic dermatitis: new insights and opportunities for therapeutic intervention. *J. Allergy Clin. Immunol.* **105**:860–876.
- Mitchell, E.B., et al. 1982. Basophils in allergen-induced patch test sites in atopic dermatitis. *Lancet*. **1**:127–130.
- Vickery, B.P. 2007. Skin barrier function in atopic dermatitis. *Curr. Opin. Pediatr.* **19**:89–93.
- Palmer, C.N., et al. 2006. Common loss-of-function variants of the epidermal barrier protein filaggrin are a major predisposing factor for atopic dermatitis. *Nat. Genet.* **38**:441–446.
- Homey, B., Steinhoff, M., Ruzicka, T., and Leung, D.Y. 2006. Cytokines and chemokines orchestrate atopic skin inflammation. *J. Allergy Clin. Immunol.* **118**:178–189.
- Spergel, J.M., et al. 1998. Epicutaneous sensitization with protein antigen induces localized allergic dermatitis and hyperresponsiveness to methacholine after single exposure to aerosolized antigen in mice. *J. Clin. Invest.* **101**:1614–1622.
- Spergel, J.M., Mizoguchi, E., Oettgen, H., Bhan, A.K., and Geha, R. 1999. Roles of T(H)1 and T(H)2 cytokines in a murine model of allergic dermatitis. *J. Clin. Invest.* **103**:1103–1111.
- Ma, W., et al. 2002. CCR3 is essential for skin eosinophilia and airway hyperresponsiveness in a murine model of allergic skin inflammation. *J. Clin. Invest.* **109**:621–628.
- Ohl, L., et al. 2004. CCR7 governs skin dendritic cell migration under inflammatory and steady-state conditions. *Immunity*. **21**:279–288.
- Robert, C., and Kupper, T.S. 1999. Inflammatory skin diseases, T cells, and immune surveillance. *N. Engl. J. Med.* **341**:1817–1828.
- Waithman, J., et al. 2007. Skin-derived dendritic cells can mediate deletion of tolerance of class I-restricted self-reactive T cells. *J. Immunol.* **179**:4535–4541.
- Merad, M., et al. 2002. Langerhans cells renew in the skin throughout life under steady-state conditions. *Nat. Immunol.* **3**:1135–1141.
- Marsland, B.J., et al. 2005. CCL19 and CCL21 induce a potent proinflammatory differentiation program in licensed dendritic cells. *Immunity*. **22**:493–505.
- Kobayashi, Y., Matsumoto, M., Kotani, M., and Makino, T. 1999. Possible involvement of matrix metalloproteinase-9 in Langerhans cell migration and maturation. *J. Immunol.* **163**:5989–5993.
- Ratzinger, G., et al. 2002. Matrix metalloproteinases 9 and 2 are necessary for the migration of Langerhans cells and dermal dendritic cells from human and murine skin. *J. Immunol.* **168**:4361–4371.
- Sawicki, G., Marcoux, Y., Sarkhosh, K., Tredget, E.E., and Ghahary, A. 2005. Interaction of keratinocytes and fibroblasts modulates the expression of matrix metalloproteinases-2 and -9 and their inhibitors. *Mol. Cell. Biochem.* **269**:209–216.
- Monteleone, G., et al. 2006. Control of matrix metalloproteinase production in human intestinal fibroblasts by interleukin 21. *Gut*. **55**:1774–1780.
- Herber, D., et al. 2007. IL-21 has a pathogenic role in a lupus-prone mouse model and its blockade with IL-21R:Fc reduces disease progression. *J. Immunol.* **178**:3822–3830.
- Distler, J.H., et al. 2005. Expression of interleukin-21 receptor in epidermis from patients with systemic sclerosis. *Arthritis Rheum.* **52**:856–864.
- Parrish-Novak, J., et al. 2000. Interleukin 21 and its receptor are involved in NK cell expansion and regulation of lymphocyte function. *Nature*. **408**:57–63.
- Brandt, K., Bulfone-Paus, S., Foster, D.C., and Ruckert, R. 2003. Interleukin-21 inhibits dendritic cell activation and maturation. *Blood*. **102**:4090–4098.
- He, R., Oyoshi, M.K., Jin, H., and Geha, R.S. 2007.



- Epicutaneous antigen exposure induces a Th17 response that drives airway inflammation after inhalation challenge. *Proc. Natl. Acad. Sci. U. S. A.* **104**:15817–15822.
41. Imai, T., et al. 1999. Selective recruitment of CCR4-bearing Th2 cells toward antigen-presenting cells by the CC chemokines thymus and activation-regulated chemokine and macrophage-derived chemokine. *Int. Immunol.* **11**:81–88.
42. Liddiard, K., et al. 2006. Interleukin-4 induction of the CC chemokine TARC (CCL17) in murine macrophages is mediated by multiple STAT6 sites in the TARC gene promoter. *BMC Mol. Biol.* **7**:45.
43. Nomura, T., et al. 2002. Interleukin-13 induces thymus and activation-regulated chemokine (CCL17) in human peripheral blood mononuclear cells. *Cytokine.* **20**:49–55.
44. Fantini, M.C., et al. 2007. IL-21 regulates experimental colitis by modulating the balance between Treg and Th17 cells. *Eur. J. Immunol.* **37**:3155–3163.
45. Anderson, A.O., and Shaw, S. 2005. Conduit for privileged communications in the lymph node. *Immunity.* **22**:3–5.
46. Brandt, K., et al. 2003. Interleukin-21 inhibits dendritic cell-mediated T cell activation and induction of contact hypersensitivity in vivo. *J. Invest. Dermatol.* **121**:1379–1382.
47. Wurster, A.L., et al. 2002. Interleukin 21 is a T helper (Th) cell 2 cytokine that specifically inhibits the differentiation of naive Th cells into interferon gamma-producing Th1 cells. *J. Exp. Med.* **196**:969–977.
48. Zeng, R., Spolski, R., Casas, E., Zhu, W., Levy, D.E., and Leonard, W.J. 2007. The molecular basis of IL-21-mediated proliferation. *Blood.* **109**:4135–4142.
49. van Leeuwen, E.M., et al. 2005. Functional re-expression of CCR7 on CMV-specific CD8+ T cells upon antigenic stimulation. *Int. Immunol.* **17**:713–719.
50. Leung, D.Y., Boguniewicz, M., Howell, M.D., Nomura, I., and Hamid, Q.A. 2004. New insights into atopic dermatitis. *J. Clin. Invest.* **113**:651–657.
51. Wenzel, J., et al. 2005. Enhanced type I interferon signalling promotes Th1-biased inflammation in cutaneous lupus erythematosus. *J. Pathol.* **205**:435–442.
52. Kasaian, M.T., et al. 2002. IL-21 limits NK cell responses and promotes antigen-specific T cell activation: a mediator of the transition from innate to adaptive immunity. *Immunity.* **16**:559–569.
53. Ozaki, K., et al. 2004. Regulation of B cell differentiation and plasma cell generation by IL-21, a novel inducer of Blimp-1 and Bcl-6. *J. Immunol.* **173**:5361–5371.
54. Kettner, A., et al. 2004. WIP regulates signaling via the high affinity receptor for immunoglobulin E in mast cells. *J. Exp. Med.* **199**:357–368.
55. Pfaffl, M.W. 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* **29**:e45.
56. Lutz, M.B., et al. 1999. An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. *J. Immunol. Methods.* **223**:77–92.
57. Pivniouk, V.I., et al. 1999. SLP-76 deficiency impairs signaling via the high-affinity IgE receptor in mast cells. *J. Clin. Invest.* **103**:1737–1743.
58. Baekkevold, E.S., et al. 2005. A role for CCR4 in development of mature circulating cutaneous T helper memory cell populations. *J. Exp. Med.* **201**:1045–1051.
59. Boes, M., et al. 2002. T-cell engagement of dendritic cells rapidly rearranges MHC class II transport. *Nature.* **418**:983–988.
60. Le, Y., et al. 2007. SOCS3 protein developmentally regulates the chemokine receptor CXCR4-FAK signaling pathway during B lymphopoiesis. *Immunity.* **27**:811–823.