

# **$\alpha 4\beta 7$ integrin is essential for contact hypersensitivity by regulating migration of T cells to skin**

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**Background:**  $\beta 7$  integrin, a cell adhesion molecule, is present in the form of  $\alpha 4\beta 7$  integrin or  $\alpha E\beta 7$  integrin.  $\alpha 4\beta 7$  integrin is expressed on most leucocytes and is essential for their migration to gut-associated lymphoid tissues by interacting with its primary ligand, mucosal addressin cell adhesion molecule-1, which is preferentially expressed in gut-associated lymphoid tissues. Although the importance of  $\alpha 4\beta 7$  integrin in intestinal inflammation has been established, its role in cutaneous inflammation remains to be elucidated.

**Objective:** We sought to investigate the role of  $\beta 7$  integrin in cutaneous inflammation.

**Methods:** We used a murine contact hypersensitivity model and examined the role of  $\beta 7$  integrin by using  $\beta 7$  integrin-deficient and  $\alpha E$  integrin-deficient mice.

**Results:**  $\beta 7$  integrin-deficient mice, not  $\alpha E$  integrin-deficient mice, are defective in contact hypersensitivity responses.  $\beta 7$  integrin deficiency does not affect irritant contact dermatitis. The distribution, migration, and function of antigen presenting cells from  $\beta 7$  integrin-deficient mice are comparable to those from wild-type mice. Moreover, sensitized  $\beta 7$  integrin-deficient T cells are able to respond to antigen stimuli *in vitro* and elicit contact hypersensitivity responses when directly injected into the skin. However, they are defective in reaching the skin under inflammatory conditions, resulting in reduced contact hypersensitivity responses when intravenously injected. Furthermore, intraperitoneal injection of anti- $\alpha 4\beta 7$  integrin neutralizing antibody elicit impaired contact hypersensitivity responses.

**Conclusion:**  $\alpha 4\beta 7$  integrin contributes to contact hypersensitivity responses by regulating T-cell migration to inflammatory skin. (J Allergy Clin Immunol 2010;■■■:■■■-■■■.)

**Key words:**  $\alpha 4\beta 7$  integrin, contact hypersensitivity responses, VCAM-1

Leukocytes recirculate through blood and secondary lymphoid organs, and they also migrate into peripheral tissues to elicit immune responses.<sup>1</sup> Aberrant leukocyte trafficking, however, leads to inflammatory and autoimmune diseases. Leukocyte trafficking is orchestrated and controlled by combinatorial inputs of adhesion and chemoattractant molecules located on both leukocytes and vascular endothelium.

Integrins, a family of cell adhesion molecules, regulate leukocyte adhesion and migration into blood vessels of lymphoid organs and at sites of inflammation. Integrins are composed of an  $\alpha$  subunit and a  $\beta$  subunit.  $\beta 7$  integrin forms heterodimer either with  $\alpha 4$  integrin or  $\alpha E$  integrin.<sup>2</sup>  $\alpha 4\beta 7$  integrin is expressed on lymphocytes,<sup>3</sup> macrophages,<sup>4</sup> mast cells,<sup>5</sup> natural killer cells,<sup>6</sup> and eosinophils,<sup>7</sup> and its ligands include mucosal addressin cell adhesion molecule-1 (MAdCAM-1)<sup>8,9</sup> and vascular cell adhesion molecule-1 (VCAM-1).<sup>10</sup> MAdCAM-1 is the main ligand of  $\alpha 4\beta 7$  integrin and is expressed on Peyer's patches of intestines, high endothelium venules of mesenteric lymph nodes, and vascular endothelial cells of gut lamina propria.<sup>11,12</sup> VCAM-1 expressed on activated vascular endothelial cells is induced by inflammation.<sup>10</sup> VCAM-1 binds to  $\alpha 4\beta 1$  integrin in addition to  $\alpha 4\beta 7$  integrin.<sup>13</sup>

$\alpha 4\beta 7$  integrin organizes migration of leukocytes to Peyer's patches and mesenteric lymph nodes by binding to MAdCAM-1. Involvement of  $\beta 7$  integrin in intestinal immunity has been extensively explored. In murine models, migration of activated CD8<sup>+</sup> T cells to the mesenteric lymph nodes and Peyer's patches after viral infection depends on  $\alpha 4\beta 7$  integrin.<sup>14</sup>  $\alpha 4\beta 7$  integrin is also important for the invasion of alloreactive donor T cells into the gut and the subsequent development of intestinal graft-versus-host disease.<sup>15</sup> In another study, pathogenic CD4<sup>+</sup> T cells use the  $\alpha 4\beta 7$  integrin/MAdCAM-1 pathway to recirculate to the chronically inflammatory small intestine in models of Crohn disease-like ileitis.<sup>16</sup> In human beings, MLN0002, a mAb targeting the  $\alpha 4\beta 7$  integrin, is effective for disease control against both ulcerative colitis<sup>17</sup> and Crohn disease.<sup>18</sup> As for  $\alpha E\beta 7$  integrin, the onset and maintenance of murine Crohn disease models depend on the colonic localization of  $\alpha E\beta 7$  integrin-expressing lamina propria CD4<sup>+</sup> T cells.<sup>19</sup>

On the other hand, little has been known about the engagements of  $\beta 7$  integrin in cutaneous inflammation.  $\beta 7$  integrin-deficient ( $\beta 7^{-/-}$ ) mice show delayed skin graft rejections,<sup>20</sup> and  $\alpha E$  integrin-deficient ( $\alpha E^{-/-}$ ) mice, in certain backgrounds, are known to develop dermatitis.<sup>21</sup> However, the mechanisms of these findings

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**Abbreviations used**

|                    |                                                 |
|--------------------|-------------------------------------------------|
| $\alpha E^{-/-}$ : | $\alpha E$ integrin-deficient                   |
| APC:               | Antigen presenting cell                         |
| $\beta 7^{-/-}$ :  | $\beta 7$ integrin-deficient                    |
| CFSE:              | Carboxyfluorescein diacetate succinimidyl ester |
| CHS:               | Contact hypersensitivity                        |
| CLA:               | Cutaneous lymphocyte-associated antigen         |
| DC:                | Dendritic cell                                  |
| DLN:               | Draining lymph node                             |
| DNBS:              | 2,4-Dinitrobenzene sulfonic acid                |
| DNFB:              | 2,4-Dinitrofluorobenzene                        |
| FACS:              | Fluorescence-activated cell sorting             |
| FITC:              | Fluorescein isothiocyanate                      |
| MAdCAM-1:          | Mucosal addressin cell adhesion molecule-1      |
| oxazolone:         | 2-Phenyl-4-ethoxymethylene oxazolone            |
| VCAM-1:            | Vascular cell adhesion molecule-1               |
| WT:                | Wild-type                                       |

were far from clear. It is well known that VCAM-1, one of the ligands for  $\alpha 4\beta 7$  integrin, is upregulated at sites of cutaneous inflammation.<sup>10</sup> Therefore, we set out to examine the role of  $\beta 7$  integrin in cutaneous immune responses by using contact hypersensitivity (CHS) models. The results of this study indicate that  $\alpha 4\beta 7$  integrin contributes to CHS responses by regulating T-cell migration to inflammatory skin.

## METHODS

### Mice

C57BL/6 mice were purchased from SLC Japan (Tokyo, Japan).  $\beta 7^{-/-}$  mice were generated as described.<sup>22</sup>  $\alpha E^{-/-}$  mice were obtained from Jackson Laboratory (Bar Harbor, Me). All mice were free of pathogenic bacteria and viruses as determined by antibody screening and routine histologic analysis of organs and tissues. All experiments were performed by using mice between 8 and 14 weeks of age. All studies and procedures were approved by the Animal Experiment Committee of the Graduate School of Medicine of the University of Tokyo guided by the Bioscience Committee of the University of Tokyo.

### CHS responses

Contact hypersensitivity responses were induced by using 2,4-dinitrofluorobenzene (DNFB) as previously described.<sup>23</sup> The volume 50  $\mu$ L 0.5% DNFB in 4:1 acetone/olive oil solution was painted on the shaved abdomens of mice on days 0 and 1. Mice were challenged on day 6 by topically applying 20  $\mu$ L 0.25% DNFB (10  $\mu$ L/site of the pinna) onto each side of the left ear. The right ear received the vehicle alone. CHS responses to 2-phenyl-4-ethoxymethylene oxazolone (oxazolone) were induced by applying 150  $\mu$ L 3% oxazolone in 3:1 alcohol/acetone solution on the shaved abdomens on day 0, and then challenged on day 7 with 20  $\mu$ L 1% oxazolone (10  $\mu$ L/site). CHS responses to fluorescein isothiocyanate (FITC) were induced by applying 400  $\mu$ L 0.5% FITC in 1:1 acetone/dibutyl phthalate to the shaved abdomen. Six days later, the CHS reactions were elicited by applying 20  $\mu$ L of the FITC solution (10  $\mu$ L/site). For all CHS experiments, baseline ear thickness was determined with a caliper (dial thickness gauge; Ozaki Manufacturing, Tokyo, Japan). Ear swelling responses were measured at 24 hours after elicitation, and the change in ear thickness from baseline measurement was computed. Each ear was measured 5 times, and the mean of these values was used. Croton oil was used to elicit irritant contact dermatitis: 15  $\mu$ L 1.0% croton oil in 4:1 acetone/olive oil solution was painted on the pinna, and 16 hours later, the change in ear thickness from baseline was measured as described for CHS responses. DNFB, oxazolone, FITC, and croton oil were purchased from Sigma-Aldrich (St Louis, Mo).

### Histologic examination

Ears were excised 24 hours after elicitation with DNFB. Excised ears were fixed in 2% paraformaldehyde and embedded. Sections, 4  $\mu$ m thick, were stained with hematoxylin and eosin. For immunohistochemistry, epidermal sheets were stained with phycoerythrin-conjugated anti-I-A<sup>b</sup> antibody (AF6-120.1; BD Pharmingen, San Diego, Calif).

### Purification of cell subsets

Lymph nodes (inguinal and axillary) and spleens were used to prepare single-cell suspensions. T cells were isolated by negative selection by using the magnetic-activated cell sorting system (pan T-cell isolation kit; Miltenyi Biotec, Bergisch Gladbach, Germany). Purity was routinely more than 95%. For obtaining dendritic cells (DCs) from skin, mouse ear skin was placed onto the 6-well plates containing RPMI 1640 medium supplemented with glutamine, antibiotics, and 10% FBS (1.5 mL/well) and incubated for 5 days at 37°C in 5% CO<sub>2</sub>. DCs from skin draining lymph nodes (DLNs) were prepared by enzymatic digestion with 1 mg/mL collagenase D (Sigma-Aldrich) and 0.2 mg/mL deoxyribonuclease (Sigma-Aldrich). After digestion, cell suspension was layered on 14.5% metrizamide (Accurate Chemical & Scientific Corp, Westburg, NY) and centrifuged. By discarding the top layer of the suspension, DCs were collected at the interface.

### Antigen-specific proliferation assay

*In vitro* proliferation to 2,4-dinitrobenzene sulfonic acid (DNBS; Sigma-Aldrich), the water-soluble analog of DNFB, was carried out as follows. In all experiments, cells were cultured in the presence of DNBS for 3 days. In some experiments, inguinal and axillary lymph node cells collected on day 1 after sensitization to DNFB were used as antigen presenting cells (APCs). Inguinal and axillary lymph node cells from unsensitized mice were also used as APCs, and they were incubated for 20 minutes at 37°C with 4 mmol/L DNBS before use. APCs ( $5 \times 10^5$ ) were then treated with 25  $\mu$ g/mL mitomycin C (Calbiochem, Darmstadt, Germany) for 25 minutes and incubated for 3 days with purified T cells harvested on day 5 after DNFB sensitization. Cultures were pulsed with 1  $\mu$ Ci/well [<sup>3</sup>H] thymidine (Amersham Bioscience, Buckinghamshire, United Kingdom). T-cell proliferation was determined in triplicate wells of 96-well plates by [<sup>3</sup>H] thymidine uptake during the last 18 hours. All experiments were performed at least 3 times.

### Ex vivo chemotaxis assay

2,4-Dinitrofluorobenzene-painted mouse ear skin was placed onto 6-well plates containing RPMI 1640 medium supplemented with glutamine, antibiotics, and 10% FBS (2 mL/well) and incubated for 24 hours at 37°C in 5% CO<sub>2</sub>. Migratory DCs were collected, stained with propidium iodide (BD Pharmingen), and FITC-conjugated CD11c antibody (HL3; BD Pharmingen). The proportion of CD11c<sup>+</sup> cells per propidium iodide-negative population was calculated by using a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ) and CellQuest software (Becton Dickinson).<sup>24</sup>

### Adoptive transfer of sensitized lymph node cells

Donor mice were sensitized with DNFB as described. On day 6, T cells were isolated from DLNs by negative selection using a pan T-cell isolation kit (Miltenyi Biotec). Naive recipient mice intravenously received  $5 \times 10^5$  T cells, which were from donor mice, in 200  $\mu$ L PBS. Eighteen hours later, mice were elicited with 0.25% DNFB, and after 24 hours, ear swelling responses were measured. As a control, unsensitized wild-type (WT) or  $\beta 7^{-/-}$  mice were painted with 0.25% DNFB on the pinna. In another experiment, sensitized  $2 \times 10^6$  T cells in 20  $\mu$ L PBS were intradermally injected into both ears using a 27-gauge needle. Recipients were immediately treated with 0.25% DNFB solution on the left ears and vehicle on the right ears. Ear swellings were measured after 24 hours.

## T-cell recruitment to CHS sites

The procedure was performed as previously described with some modification.<sup>25</sup> DNFB-primed T cells were prepared as described and labeled with 1  $\mu$ mol/L carboxyfluorescein diacetate succinimidyl ester 5-chloromethyl-fluorescein diacetate (CFSE; Molecular Probes, Eugene, Ore). Naive recipient mice intravenously received  $5 \times 10^6$  labeled T cells and were challenged with 0.25% DNFB solution on the left ear and vehicle on the right ear. Twelve hours after the transfer, the dorsal portions of dermis were collected from each ear. Single cell suspensions were prepared from the ear tissues as previously described.<sup>26</sup> CFSE<sup>+</sup> cells in a total of 100,000 nucleated cells were enumerated by flow cytometer.

## Blocking of CHS responses by using antibodies

Anti- $\alpha 4\beta 7$  integrin (DATK 32), anti- $\beta 7$  integrin (FIB27), and isotype-matched control (R35-95) antibodies were obtained from BD Pharmingen. Anti- $\alpha 4$  integrin antibody (PS/2) was obtained from American Type Culture Collection (CRL-1911; Manassas, Va). WT mice were sensitized with DNFB as described. On day 6, each antibody (65  $\mu$ g/body) was administered intraperitoneally to sensitized mice. Immediately after the injection, mice were elicited with 0.25% DNFB, and after 24 hours, ear swelling responses were measured.

## Statistics

The Student *t* test was used to assess differences between 2 groups. A *P* value of less than .05 was considered significant. When multiple groups were compared, 1-way ANOVA followed by the Turkey-Kramer multiple comparisons test were performed. A *P* value less than .05 was considered significant.

## RESULTS

### $\beta 7^{-/-}$ mice exhibit impaired CHS responses

To evaluate the roles of  $\beta 7$  integrin in cutaneous immune responses, we examined CHS responses in  $\beta 7^{-/-}$  mice. The  $\beta 7^{-/-}$  and WT mice were challenged with DNFB, oxazolone, or FITC after sensitization, and ear swelling was measured 24 hours after elicitation. The increase of ear thickness was significantly lower in  $\beta 7^{-/-}$  mice than in WT mice when DNFB was used as hapten ( $P < .01$ ; Fig 1, A). Similar results were obtained when we used different haptens such as FITC ( $P < .01$ ; Fig 1, B) and oxazolone ( $P < .01$ ; Fig 1, C). CHS responses were also evaluated histopathologically. The ear swelling and cellular infiltration were not prominent in  $\beta 7^{-/-}$  mice compared with WT mice (Fig 1, E). The profiles of infiltrating cells were similar in both strains, which mainly consisted of polymorphonuclear cells, lymphoid cells, and macrophages. To exclude the possibility that nonspecific inflammatory responses are exaggerated in  $\beta 7^{-/-}$  mice, immune responses to irritants were examined. Croton oil was applied to ears of  $\beta 7^{-/-}$  and WT mice, and ear thickness was measured after 16 hours. The increase of ear thickness was similar in both  $\beta 7^{-/-}$  and WT mice (Fig 1, D). These results demonstrate that CHS responses are impaired in  $\beta 7^{-/-}$  mice, but that those mice respond normally to irritants.

### CHS responses in $\alpha E^{-/-}$ mice are not impaired

$\beta 7^{-/-}$  mice lack both  $\alpha 4\beta 7$  and  $\alpha E\beta 7$  integrins because  $\beta 7$  integrin forms heterodimer with either  $\alpha 4$  or  $\alpha E$  integrin. To investigate which heterodimer is critical for CHS responses,  $\alpha E^{-/-}$  mice were challenged with various haptens.  $\alpha E^{-/-}$  mice are known to lack solely  $\alpha E\beta 7$  integrin and retain  $\alpha 4\beta 7$  integrin.<sup>27</sup> The increase of ear thickness in  $\alpha E^{-/-}$  mice was comparable to

that in WT mice when those mice were challenged with DNFB (Fig 2, A). Similar results were obtained when either FITC (Fig 2, B) or oxazolone (Fig 2, C) was used as hapten. As expected, the increase of ear thickness was similar in both  $\alpha E^{-/-}$  and WT mice when those mice were applied with croton oil (Fig 2, D). Therefore, the impairment of CHS responses observed in  $\beta 7^{-/-}$  mice is mainly a result of  $\alpha 4\beta 7$  integrin deficiency.

### $\beta 7$ integrin deficiency does not affect migration of APCs

Contact hypersensitivity responses involve a sensitization phase and a challenge phase. In the sensitization phase, hapten-loaded APCs migrate to the lymph nodes and prime hapten-specific T cells. To determine whether  $\beta 7$  integrin regulates the sensitization phase, we first examined the number and distribution of DCs in the skin. Epidermal sheets from ears were stained with anti-I-A<sup>b</sup> antibody, and I-A<sup>b</sup> cells were enumerated. The number of I-A<sup>b</sup> cells was similar between  $\beta 7^{-/-}$  and WT mice (Fig 3, A). Moreover, the density, morphology, and distribution of DCs were comparable between those strains (Fig 3, B and C). Epidermal sheets were also stained with anti- $\beta 7$  integrin antibody. I-A<sup>b</sup> cells in the epidermis from WT mice expressed  $\beta 7$  integrin, whereas those from  $\beta 7^{-/-}$  mice did not (data not shown).

To examine whether antigen-bearing DCs appropriately leave the skin in  $\beta 7^{-/-}$  mice, ears were floated on the medium, and the cells were collected from the medium. When ears painted with DNFB were used, the proportion of CD11c<sup>+</sup> cells per all collected cells was not significantly different between WT and  $\beta 7^{-/-}$  mice (Fig 3, D). Thus, the exodus of DCs from the skin is not impaired despite the lack of  $\beta 7$  integrin.

After migrating from the skin, DCs enter afferent lymph ducts and reach DLNs by lymph flow. To evaluate the lymph flow, Evans blue dye was injected into footpads, and the amount of dye reaching the DLNs was measured. The concentration of Evans blue dye was not decreased in  $\beta 7^{-/-}$  mice, suggesting lymph flow is not perturbed in  $\beta 7^{-/-}$  mice (data not shown).

To evaluate whether APCs from  $\beta 7^{-/-}$  mice uptake antigen and migrate from the skin to the DLNs, FITC was applied to the abdomen, and after 24 hours, FITC<sup>+</sup> I-A<sup>b</sup> APCs in the DLNs were evaluated by fluorescence-activated cell sorting (FACS). In WT mice,  $71.8\% \pm 2.0\%$  of I-A<sup>b</sup> cells were positive for FITC. Similarly,  $69.5\% \pm 5.0\%$  of I-A<sup>b</sup> cells were positive for FITC in  $\beta 7^{-/-}$  mice (Fig 4, A). These results indicate that antigens painted on the skin of  $\beta 7^{-/-}$  mice are taken up by APCs, which then migrate in normal numbers to DLNs.

### APCs lacking $\beta 7$ integrin can normally prime T cells

It has been shown that DCs existing in the skin reach the DLNs 3 to 8 hours after transdermal stimulation.<sup>28</sup> To determine whether APCs in the  $\beta 7^{-/-}$  mice are functionally normal, DLNs from WT and  $\beta 7^{-/-}$  mice 24 hours after painting with DNFB were collected, treated with mitomycin C, and then used as APCs. These APCs were cultured with DNFB-sensitized lymph node T cells from WT mice. APCs from  $\beta 7^{-/-}$  mice were as competent at priming T cells as those from WT mice (Fig 4, B). This result demonstrates that  $\beta 7$  integrin deficiency does not influence antigen-presenting capacity of APCs.

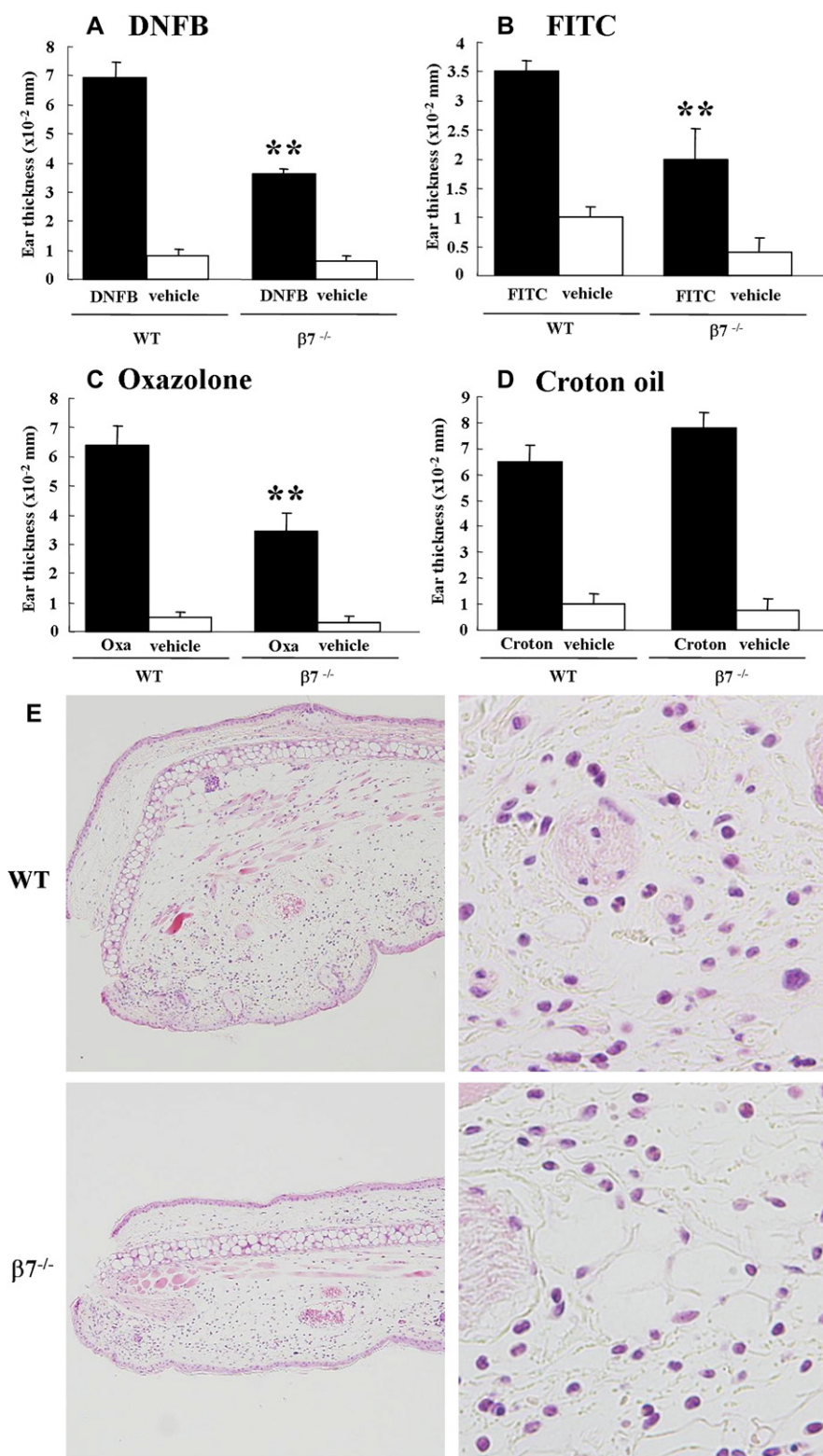


**FIG 1.**  $\beta 7^{-/-}$  mice exhibit impaired CHS responses. CHS responses with DNFB (A), FITC (B), and oxazolone (C). D, Irritant dermatitis with croton oil. Values are means  $\pm$  SEMs (n = 6-15 per group). \*\* $P < .01$  versus WT mice. E, Hematoxylin and eosin staining of sections from ears of WT mice and  $\beta 7^{-/-}$  mice with DNFB elicitation. Original magnification,  $\times 100$  (left panel);  $\times 800$  (right panel). Oxa, oxazolone.

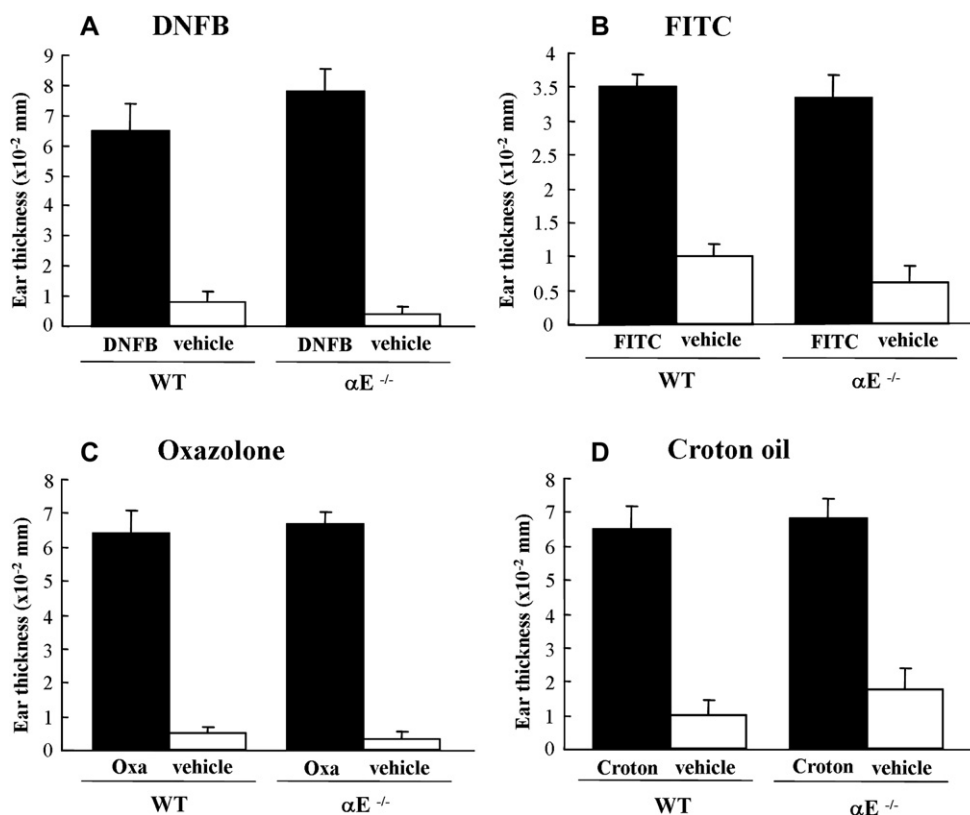


**FIG 2.** CHS responses in  $\alpha E^{-/-}$  mice are not impaired. CHS responses with DNFB (A), FITC (B), and oxazolone (C). D, Irritant dermatitis with croton oil. Values are means  $\pm$  SEMs (n = 6–8 per group).

### $\beta 7$ integrin-deficient T cells show normal hapten-specific proliferation

We next examined whether  $\beta 7$  integrin-deficient T cells were able to proliferate after antigen stimulation. T cells were purified from DLNs from WT and  $\beta 7^{-/-}$  mice 5 days after sensitization. Those T cells were cultured with DNBS-conjugated WT lymph node cells treated with mitomycin C.  $\beta 7$  integrin-deficient T cells proliferated equally to WT T cells in response to antigen stimulation (Fig 4, C). These results indicate that the proliferation of hapten-specific T cells in the DLNs is not disturbed in the absence of  $\beta 7$  integrin. Collectively, we have concluded that the sensitization phase of CHS is intact in  $\beta 7$  integrin deficiency.

### Hapten-specific $\beta 7$ integrin-deficient T cells induce normal CHS responses when directly injected into the skin

Next we investigated whether  $\beta 7$  integrin-deficient T cells could induce cutaneous inflammation *in situ*. To test this, T cells from the DLNs of DNFB-sensitized WT or  $\beta 7^{-/-}$  mice were intradermally injected into the ear of WT recipients, and those mice were challenged with DNFB. The intensity of ear swelling responses induced by sensitized  $\beta 7$  integrin-deficient T cells was comparable to that induced by WT T cells (Fig 5, A). In addition, T cells from DNFB-sensitized WT mice were intradermally injected into the ear of either WT or  $\beta 7$  integrin-deficient recipients. WT and  $\beta 7$  integrin-deficient recipients showed similar degrees of ear swelling (Fig 5, A).

These results indicate that sensitized  $\beta 7$  integrin-deficient T cells can elicit CHS if they exist in target skin, and  $\beta 7$  integrin-deficient recipients show normal CHS responses if they have sensitized T cells in target skin.

### $\beta 7$ integrin is required for T-cell trafficking to the inflammatory skin

At the challenge phase of CHS, ear swelling is initiated by antigen-specific T cells followed by the recruitment of additional cells.<sup>29</sup> To examine the trafficking of T cells to the skin, we adoptively transferred T cells from DNFB-sensitized WT or  $\beta 7^{-/-}$  mice into WT or  $\beta 7$  integrin-deficient recipients, and recipients were then challenged with DNFB. Although WT T cells induced CHS in both WT and  $\beta 7$  integrin-deficient recipients to a similar extent,  $\beta 7$  integrin-deficient T cells failed to elicit CHS responses even in WT recipients (Fig 5, B). Considering that the sensitization phase of CHS is normal in  $\beta 7^{-/-}$  mice and that the challenge phase of CHS is sufficiently induced when  $\beta 7$  integrin-deficient T cells exist in target skin, these results suggest that sensitized  $\beta 7$  integrin-deficient T cells are defective in trafficking to the skin.

To verify that hypothesis, T cells from DNFB-sensitized WT or  $\beta 7^{-/-}$  mice were labeled with CFSE, and those T cells were injected intravenously into WT recipients that were then immediately challenged with DNFB. Sensitized  $\beta 7$  integrin-deficient T cells migrated poorly to the ear compared with WT T cells (Fig 6, A). On the other hand, sensitized  $\beta 7$  integrin-deficient T cells migrated to spleens and DLNs more than WT T cells (Fig 6, B and C) and distributed equally to blood (Fig 6, D). These

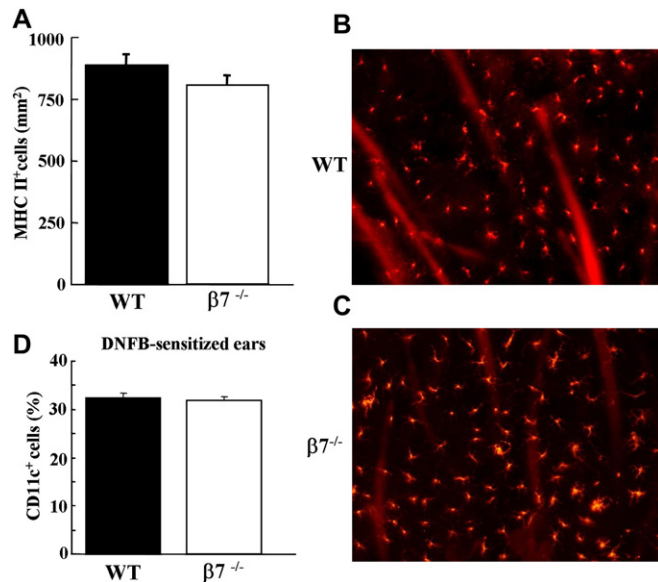


**FIG 3.** The number, distribution, and lymph flow of APCs from  $\beta 7^{-/-}$  mice are comparable to those from WT mice. **A**, Cell counts for I-A<sup>b</sup> cells per within epidermis. **B and C**, Epidermal sheets were stained with anti-I-A<sup>b</sup> antibody. Values are means  $\pm$  SEMs (n = 5 per group). **D**, Ex vivo chemotaxis assay. Values are means  $\pm$  SEMs (n = 6 per group).

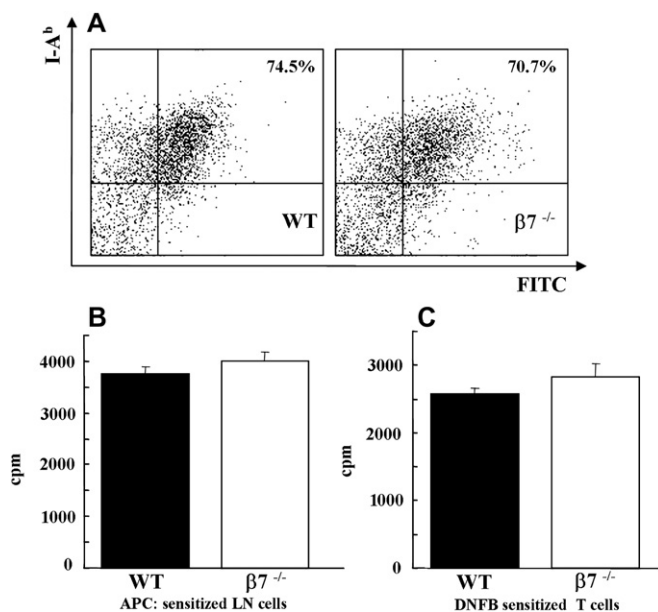


**FIG 4.** The migration and function of APCs from  $\beta 7^{-/-}$  mice are comparable to those from WT mice. **A**, The number shows that the percentage of FITC<sup>+</sup> cells within I-A<sup>b</sup> cell populations in DLNs. Shown are representative FACS plots. **B**, APCs obtained from DLNs of sensitized WT or  $\beta 7^{-/-}$  mice were cultured with DNFB-sensitized WT T cells. **C**, APCs obtained from DLNs of unsensitized WT mice were treated with mitomycin C and cultured with DNFB-sensitized WT or  $\beta 7^{-/-}$  T cells. **B and C**, Values are means  $\pm$  SEMs. cpm, Counts per minute; LN, lymph node.

results demonstrate that  $\beta 7$  integrin is important for sensitized T cells to reach the skin.

Finally, we used a blocking antibody to confirm the role of  $\beta 7$  integrin in CHS. DNFB-sensitized WT mice were intraperitoneally injected with anti- $\alpha 4$ , anti- $\beta 7$ , anti- $\alpha 4\beta 7$  integrin neutralizing

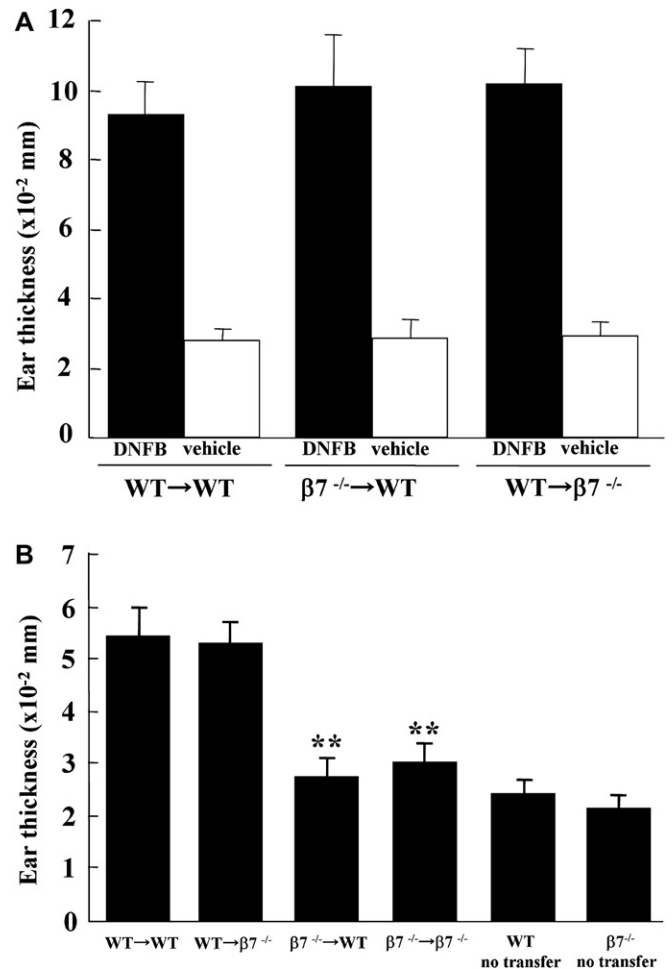


**FIG 5.** Hapten-specific  $\beta 7^{-/-}$  T cells induce normal CHS responses when directly injected to the skin, whereas ear swelling is reduced when T cells from sensitized  $\beta 7^{-/-}$  mice are intravenously injected into the recipient mice. **A**, Ear swelling after intradermal injection with sensitized T cells and application with DNFB. Values are means  $\pm$  SEMs (n = 7 per group). **B**, Adoptive transfer. Unsensitized mice, which were painted with DNFB on the pinna, were used as controls (WT no transfer and  $\beta 7^{-/-}$  no transfer). Values are means  $\pm$  SEMs (n = 7-8 per group). \*\* $P < .01$  versus WT→WT.

antibody, or control antibody. Immediately after the injection, mice were challenged with DNFB. The increase of ear thickness in mice treated with anti- $\alpha 4$ , anti- $\beta 7$ , or anti- $\alpha 4\beta 7$  integrin antibody were similarly reduced compared with that in mice treated with control antibody (Fig 7). The cellular infiltration was not prominent in groups treated with anti- $\alpha 4$ , anti- $\beta 7$ , or anti- $\alpha 4\beta 7$  integrin neutralizing antibody (data not shown). The profiles of infiltrating cells were similar in all groups (data not shown). These results confirm that  $\alpha 4\beta 7$  integrin is essential for the challenge phase of CHS responses.

## DISCUSSION

Rapid recruitment of lymphocytes to sites of inflammation is crucial to mount an efficient immune response.  $\alpha 4\beta 7$  integrin is well known for lymphocyte migration into gut-associated lymphoid tissue. Here, we show that  $\alpha 4\beta 7$  integrin is also essential for lymphocyte migration to the skin. This is the first demonstration of the direct participation of  $\alpha 4\beta 7$  integrin in cutaneous immunity *in vivo*.

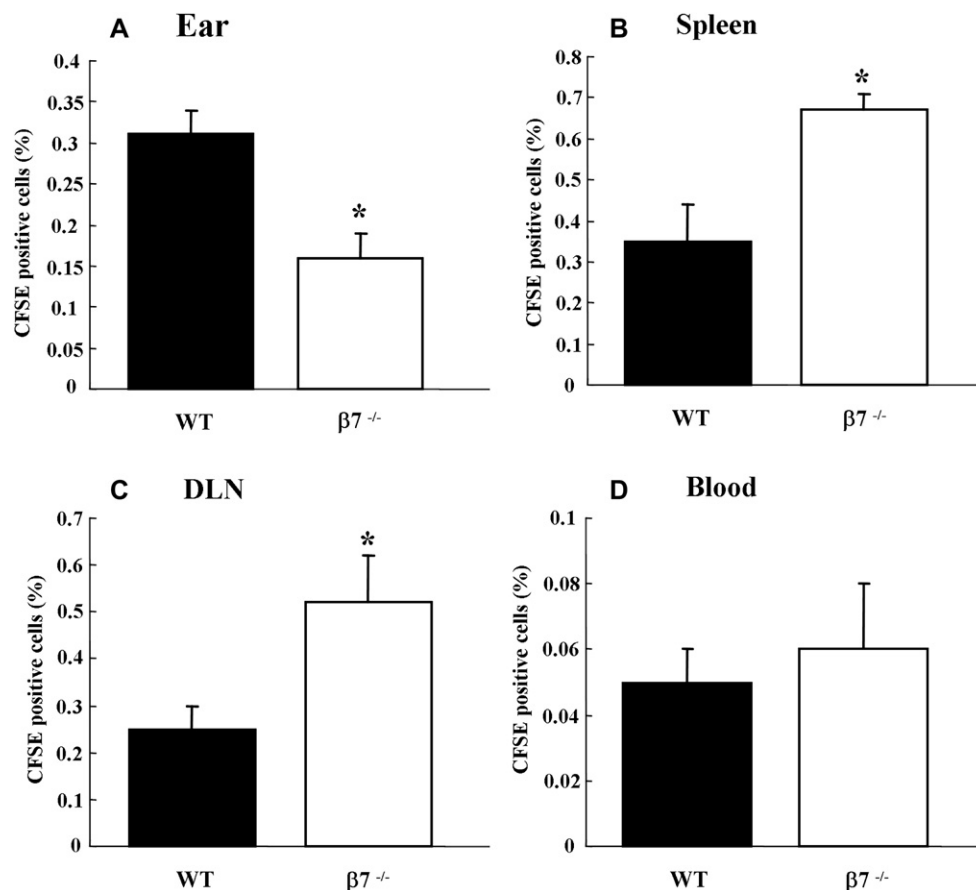


**FIG 6.** T cells from sensitized  $\beta 7^{-/-}$  mice are impaired in reaching the skin, and instead they accumulate in spleen and DLNs. Single-cell suspensions were prepared from ears (A), spleens (B), DLNs (C), and blood (D) for FACS analysis. Values are means  $\pm$  SEMs ( $n = 5-6$  per group). \* $P < .05$  versus WT.

Our current results demonstrate that  $\beta 7$  integrin deficiency causes impaired hapten-specific CHS responses (Fig 1, A-C). The CHS responses were reduced in all haptens we examined, such as DNFB, FITC, and oxazolone. In addition to  $\alpha 4\beta 7$  integrin,  $\beta 7^{-/-}$  mice lack  $\alpha E\beta 7$  integrin. Previous reports have shown that  $\alpha E\beta 7$  integrin may play a role in infiltrating T cells in the epidermis of psoriasis<sup>30</sup> and atopic dermatitis.<sup>31</sup> However, most of the infiltrating  $CD3^{+}$  cells are cutaneous lymphocyte-associated antigen (CLA)<sup>low</sup> $\alpha E\beta 7^{low}$  in patch test reaction,<sup>31</sup> which resembles contact hypersensitivity. In this study,  $\alpha E^{-/-}$  mice showed normal CHS responses. These results suggest that lack of  $\alpha 4\beta 7$  integrin, possibly not  $\alpha E\beta 7$  integrin, is responsible for the reduced CHS responses in  $\beta 7^{-/-}$  mice.

In an irritant dermatitis model using croton oil, ear swelling was not reduced in  $\beta 7^{-/-}$  mice (Fig 1, D). It has been shown that most of infiltrating cells in irritant dermatitis are neutrophils<sup>32</sup> and that  $\alpha 2\beta 1$ ,<sup>33,34</sup>  $\alpha L\beta 2$ , and  $\alpha M\beta 2$  integrins<sup>35</sup> are involved in recruiting neutrophils. Moreover, neutrophils do not, under most circumstances, express detectable levels of  $\alpha 4\beta 7$  integrin.<sup>36</sup> Thus, our results confirm that neutrophil migration to the skin is independent of  $\beta 7$  integrin.

During the sensitization phase of CHS responses, hapten-loaded APCs migrate to the lymph nodes and prime hapten-specific T cells. Langerhans cells, which are DCs resident in the epidermis, bind to keratinocytes via E-cadherin.<sup>37</sup> Deficiency of

$\beta 7$  integrin could impair the retention of DCs and reduce the number of DCs in the epidermis. Our current results showed that in  $\beta 7^{-/-}$  mice, the number of I-A<sup>b+</sup> DCs in the epidermis was not significantly reduced compared with that in WT mice (Fig 3, A). These results are compatible with the previous report showing that anti- $\alpha 4$  integrin antibody does not influence migration of epidermal DCs from the epidermis.<sup>38</sup> It is also known that  $\alpha E$  integrin deficiency does not reduce the number of epidermal Langerhans cells.<sup>39</sup> Collectively, it is unlikely that  $\beta 7$  integrin deficiency affects DC retention in the epidermis.

DCs start to migrate from the skin and upregulate expression of CCR7 after they uptake antigens. These DCs migrate into the afferent lymph ducts, expressing CC chemokine ligand 21, which is a ligand for CCR7. These DCs presumably consist of various subsets including Langerhans cells and dermal DCs. Langerhans cells have long been believed to be responsible for CHS initiation as APCs.<sup>40,41</sup> However, recent studies show that Langerhans cell depletion in mice does not significantly affect CHS responses, indicating dispensability of Langerhans cells and contribution of dermal DCs to the CHS induction.<sup>42-44</sup>  $\alpha 4\beta 1$  integrin and  $\alpha 5\beta 1$  integrin are known to play a role in motility of DCs.<sup>45,46</sup> To examine the role of  $\beta 7$  integrin for DC retention, DNFB-painted ears were floated on the medium, and the cells moving to the medium were collected. In this setting, we could evaluate both Langerhans cells and dermal DCs. The

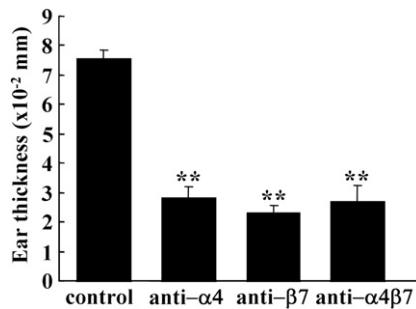


**FIG 7.** Ear swelling is reduced when sensitized mice are administered with neutralizing mAbs to  $\alpha 4$ ,  $\beta 7$ , or  $\alpha 4\beta 7$  integrins. Results are expressed as means  $\pm$  SEMs ( $n = 6-9$  per group). \*\* $P < .01$  versus control antibody.

numbers of DCs from the ear explants of WT mice and  $\beta 7^{-/-}$  mice were not significantly different (Fig 3, D), indicating that  $\beta 7$  integrin-deficient DCs are able to leave the skin as efficiently as WT DCs.

After migrating from the skin, DCs enter lymph ducts and reach the DLNs through the lymph flow. Lymph flow was not disturbed in  $\beta 7^{-/-}$  mice when we measured the dye content in DLNs after Evans blue dye injection (data not shown). Moreover, measurement of FITC<sup>+</sup> DCs in DLNs after cutaneous FITC application shows that the migration of DCs in DLNs was not altered in  $\beta 7^{-/-}$  mice (Fig 4, A). These results indicate that DCs from  $\beta 7^{-/-}$  mice are able to uptake antigens and migrate from the skin to the DLNs.

Although the interaction between lymphocyte function-associated antigen-1 ( $\alpha L\beta 2$  integrin) expressed by T cells and intercellular adhesion molecule-1 expressed by DCs is important for antigen presentation,<sup>47</sup> the role of  $\beta 7$  integrin in this process remains unknown. In an *in vitro* proliferation assay, sensitized T-cell proliferation was comparable when we used APCs either from WT mice or from  $\beta 7^{-/-}$  mice (Fig 4, B). Signal via  $\alpha 4\beta 7$  integrin activates signal transducers such as p125<sup>FAK</sup> in B cells.<sup>48</sup> p125<sup>FAK</sup> on T-lymphoblastic cells is tyrosine phosphorylated by  $\alpha 4\beta 1$  and  $\alpha 5\beta 1$  integrin engagement.<sup>49</sup> Therefore,  $\beta 7$  integrin deficiency might disturb activation and proliferation of T cells. However, T cells existing in DLNs from  $\beta 7^{-/-}$  mice exhibited normal proliferation in response to antigens (Fig 4, C). These results suggest that the sensitization phase of CHS response is not affected by  $\beta 7$  integrin deficiency.

We further investigated whether sensitized T cells in DLNs were actually able to induce ear swelling *in situ*. When sensitized T cells were subcutaneously injected, those from  $\beta 7^{-/-}$  mice induced ear swelling similar to those from WT mice (Fig 5, A). Recipient  $\beta 7^{-/-}$  mice were also able to mount the challenge phase of CHS responses when sensitized WT T cells were either subcutaneously or intravenously transferred (Fig 5). These results suggest that the reduction of CHS responses observed in  $\beta 7^{-/-}$  mice is caused by disturbed trafficking of activated T cells. Consistent with this hypothesis, ear swelling of WT mice intravenously transferred with sensitized  $\beta 7^{-/-}$  T cells was significantly reduced compared with that of mice transferred with sensitized WT T cells (Fig 5, B). Because we already showed that these sensitized T cells from  $\beta 7^{-/-}$  mice proliferated normally and induced comparable ear swelling if they were directly injected into the skin, this finding suggests that  $\beta 7$  integrin-deficient activated T cells are defective in migrating from blood to the ear.

This idea was further supported by the finding that CFSE-labeled sensitized T cells from  $\beta 7^{-/-}$  mice were inefficient in migrating to the challenged ear and that they rather trafficked to spleens and DLNs (Fig 6, A-C).

The impaired trafficking of  $\beta 7^{-/-}$  T cells suggests that  $\beta 7$  integrin interacts with its ligands at the sites of the cutaneous vasculature. The main ligand of  $\alpha 4\beta 7$  integrin is MAdCAM-1, which is mainly expressed in intestinal areas. There has been no report showing that MAdCAM-1 is expressed on the vessels in the skin. Consistent with this, we were also unable to detect the expression of MAdCAM-1 on cutaneous vessels after the elicitation (data not shown). By contrast, expression of VCAM-1, another ligand of  $\alpha 4\beta 7$  integrin, is strongly induced on endothelial cells in inflammatory skin diseases including CHS.<sup>50,51</sup> VCAM-1 also binds to  $\alpha 4\beta 1$  integrin. The interactions between VCAM-1 and  $\alpha 4\beta 1$  integrin is essential for lymphocyte tethering and rolling.<sup>52</sup> In previous reports, lymphocyte migration to the inflammatory skin was impaired by  $\alpha 4$  integrin blockade that can disturb the function of both  $\alpha 4\beta 1$  and  $\alpha 4\beta 7$  integrins.<sup>53-55</sup> Therefore, it is possible that both  $\alpha 4\beta 1$  and  $\alpha 4\beta 7$  integrins contribute to lymphocyte migration to the inflammatory skin in those settings.

The majority of activated T cells in the spleen, DLNs,<sup>56</sup> and peripheral blood<sup>57</sup> express high levels of  $\alpha 4\beta 7$  integrin. However, Campbell and Butcher<sup>58</sup> have shown that CD4<sup>+</sup> cells in DLNs downregulate  $\alpha 4\beta 7$  integrin after systemic immunization. In our experiments, we have used epicutaneous immunization, which might explain why  $\alpha 4\beta 7$  integrin is important in our study. In another human study, the frequency of  $\alpha E\beta 7^{+}$  T cells in skin blister was slightly increased, whereas that of  $\alpha 4\beta 7^{+}$  T cells was rather decreased compared with peripheral blood.<sup>57</sup> However,  $\alpha 4\beta 7$  integrin expression levels in T cells in CLA<sup>low/intermediate</sup> T cells in skin blister were comparable to those in peripheral blood. Considering that the considerable number of infiltrating cells remained CLA<sup>low/intermediate</sup> in skin blister in that study, it is possible that  $\alpha 4\beta 7$  integrin was required for those CLA<sup>low/intermediate</sup> T cells to migrate to skin. In another study, most of the infiltrating CD3<sup>+</sup> cells in patch test reaction areas were CLA<sup>low</sup> $\alpha E\beta 7^{low}$ .<sup>31</sup> It is possible that these CLA<sup>low</sup> $\alpha 4\beta 7^{high}$  CD3<sup>+</sup> cells used  $\alpha 4\beta 7$  integrin for skin infiltration. Taken together, our results indicate that  $\alpha 4\beta 7$  integrin expressed on activated T cells in the peripheral blood and VCAM-1 expressed on endothelial cells under cutaneous inflammation interact each other and that their interactions promote the CHS responses through regulating T-cell migration to the skin.

To date,  $\alpha L\beta 2$  and  $\alpha 4\beta 1$  integrin, which are ligands of intercellular adhesion molecule-1 and VCAM-1, respectively, have been identified as the major integrins that are essential for CHS responses. In the results of the current study,  $\alpha 4\beta 7$  integrin, which is thought to function predominantly in the intestine, is identified as another critical integrin that regulates CHS responses in our mouse model. Blockade of  $\beta 7$  integrin is already known to be effective for treating of graft-versus-host disease<sup>59</sup> and Crohn disease.<sup>18</sup> The role of  $\alpha 4\beta 7$  integrin in human skin is still unknown; however, our results suggest that targeting  $\beta 7$  integrin either independently or in combination of other integrins is another option for treating inflammatory skin disorders such as CHS.

**Clinical implications:  $\alpha 4\beta 7$  integrin could be a novel target for inflammatory skin disorders such as contact hypersensitivity responses by limiting T-cell migration to the inflammatory skin.**

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