Lymphatic Dysfunction Impairs Antigen-Specific Immunization, but Augments Tissue Swelling Following Contact with Allergens

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The lymph transports tissue-resident dendritic cells (DCs) to regional lymph nodes (LNs), having important roles in immune function. The biological effects on tissue inflammation following lymphatic flow obstruction *in vivo*, however, are not fully known. In this study, we investigated the role of the lymphatic system in contact hypersensitivity (CHS) responses using *k-cyclin* transgenic (kCYC$^{+/-}$) mice, which demonstrate severe lymphatic dysfunction. kCYC$^{+/-}$ mice showed enhanced ear swelling to both DNFB and FITC, as well as stronger irritant responses to croton oil compared with wild-type littermates. Consistently, challenged ears of kCYC$^{+/-}$ mice exhibited massive infiltrates of inflammatory cells. In contrast, DC migration to regional LNs, drainage of cell-free antigen to LNs, antigen-specific IFN-γ production, and lymphocyte proliferation were impaired during the sensitization phase of CHS in kCYC$^{+/-}$ mice. Transfer experiments using lymphocytes from sensitized mice and real-time PCR analysis of cytokine expression using challenged ear revealed that ear swelling was enhanced because of impaired lymphatic flow. Collectively, we conclude that insufficient lymphatic drainage augments apparent inflammation to topically applied allergens and irritants. The findings add insight into the clinical problem of allergic and irritant contact dermatitis that commonly occurs in humans with peripheral edema of the lower legs.

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INTRODUCTION

Lymphedema is a condition caused by damaged lymphatics resulting in accumulation of lymph fluid and tissue swelling. It is common in the legs of older individuals and in the arms of women following breast cancer surgery. Lymphedema is associated with a number of complications, including infections with bacteria and fungi. In rare cases, lymphedema may be complicated by the development of angiosarcoma (Stewart and Treves, 1948; Ruocco *et al.*, 2001), squamous cell carcinoma (Epstein and Mendelsohn, 1984; Furukawa *et al.*, 2002), and lymphoma (d'Amore *et al.*, 1990; Dargent *et al.*, 2005). These phenomena may be due to reduced tissue immune surveillance secondary to lymphatic dysfunction. Indeed, lymphatic vessels are critical for transporting tissue-resident dendritic cells (DCs), as well as interstitial fluid to the lymph nodes (LNs), having important roles in immunity against infectious agents and malignancy (Kaplan *et al.*, 2005). Thus far, very little is known about how immune cells traffic and how immune responses may be altered in the setting of lymphatic dysfunction.

Contact hypersensitivity (CHS) is an experimental model for the study of antigen-specific, T-cell-mediated immune responses (Macher and Chase, 1969). CHS responses comprise: (1) a sensitization phase, when an antigen is first presented to naive T cells in the regional LNs, and (2) an elicitation phase, when antigen-specific memory T cells get activated and release cytokines that attract other inflammatory cells to the exposed site, dilate cutaneous blood vessels, and cause dermal edema (Hopkins and Clark, 1995). It is widely accepted that antigen-presenting cells migrate to LNs and present antigens to naive T cells in the sensitization phase. It is unknown how lymphatic dysfunction affects CHS responses.

Specific markers for lymphatic endothelium have been reported, such as vascular endothelial growth factor receptor-3 (VEGFR-3) (Jussila *et al.*, 1998; Dupin *et al.*, 1999), podoplanin (Breiteneder-Geleff *et al.*, 1999), and lymphatic vessel endothelial hyaluronan receptor-1 (Hong *et al.*, 2004). Specific identification of lymphatic endothelial cells had led

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Abbreviations: CHS, contact hypersensitivity; DC, dendritic cell; kCYC$^{+/-}$, k-cyclin transgenic; LN, lymph node; MHC, major histocompatibility complex; PE, phycoerythrin; VEGFR-3, vascular endothelial growth factor receptor-3; WT, wild type

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to a variety of important studies on structure and function of lymphatic vessels in both normal and disease states. We recently generated transgenic mice expressing the Kaposi's sarcoma-associated herpesvirus latent-cycle gene, *k-cyclin*, under the control of the VEGFR-3 promoter (Sugaya *et al.*, 2005). In Kaposi's sarcoma, this viral gene is expressed by lymphatic endothelial cells and probably contributes to edema within lesions (Davis *et al.*, 1997; Reed *et al.*, 1998). Interestingly, most *k-cyclin* transgenic (kCYC$^{+/-}$) mice developed progressive accumulation of chylous pleural fluid. In skin, dermal edema was detected by magnetic resonance imaging (Sugaya *et al.*, 2005). In addition, lymphatic drainage of injected contrast dyes was markedly impaired in transgenic mice. Using these mice, we investigated the role of the lymphatic system in CHS responses in this study.

RESULTS

Augmented ear swelling in kCYC$^{+/-}$ mice

We first investigated whether CHS responses were impaired in kCYC$^{+/-}$ mice, which demonstrate markedly impaired lymphatic drainage (Sugaya *et al.*, 2005). When the mice were sensitized with 0.5 or 0.1% DNFB, ear swelling was significantly augmented in kCYC$^{+/-}$ mice compared with wild-type (WT) mice (Figure 1a and b). Similar results were obtained when we used FITC (Figure 1c), showing that different antigens could induce augmented ear swelling in kCYC$^{+/-}$ mice. Nonimmunized mice and FITC-challenged mice that had been sensitized with DNFB did not show CHS responses (Figure 1a and data not shown).

Enhanced cellular infiltration in the challenged ear of kCYC$^{+/-}$ mice

We also evaluated CHS responses histopathologically. There were no differences between ears from kCYC$^{+/-}$ mice and WT mice before treatment (Figure 1d). Ear swelling and cellular infiltration 24 hours after challenge with either DNFB (Figure 1d) or FITC (data not shown) were prominent in kCYC$^{+/-}$ mice compared with WT mice. Edema and dilated vessels in the ear from kCYC$^{+/-}$ mice suggested impaired lymphatic flow in these mice. There were more infiltrating cells, such as mononuclear cells, eosinophils, and major histocompatibility complex (MHC) class II^{+} DCs in kCYC$^{+/-}$ mice compared with WT mice (Figure 1e).

Impaired migration of skin-derived DCs into draining LNs of kCYC$^{+/-}$ mice during the sensitization phase of CHS

To elucidate the mechanism of augmented ear swelling in kCYC$^{+/-}$ mice, each step involved in the generation of CHS responses was examined. We first studied migration of antigen-bearing DCs from skin to regional LNs. Untreated epidermal sheets contained equal numbers of DCs (WT, $752 \pm 24/mm^2$ vs. kCYC$^{+/-}$, $806 \pm 43/mm^2$, $n = 5$). The shape and distribution of epidermal DCs (Langerhans cells) were similar in WT and kCYC$^{+/-}$ mice (Figure 2a). To count draining DCs in LNs, inguinal LN cells were harvested 24 or 48 hours after applying 0.5% FITC on shaved abdominal skin. Inguinal LN cells from untreated mice were also obtained. After applying FITC, the number of DCs and antigen-bearing DCs in draining LNs in WT mice increased, as expected (Figure 2b and c). In contrast, migration of antigen-bearing DCs in kCYC$^{+/-}$ mice was almost completely abrogated. Similar results were obtained when DCs were labeled by anti-CD11c mAb (data not shown). The results suggest that DCs cannot migrate from the skin to draining LNs when lymphatic flow is impaired. We detected almost no antigen-bearing DCs in the spleen after sensitization in both WT and kCYC$^{+/-}$ mice (data not shown). Interestingly, the numbers of DCs in kCYC$^{+/-}$ mice were significantly decreased compared with WT mice without stimuli (Figure 2c), which suggests that lymphatic dysfunction in kCYC$^{+/-}$ mice may decrease the steady-state migration of DCs (Ruedl *et al.*, 2000; Henri *et al.*, 2001; Ohl *et al.*, 2004).

Impaired proliferation of lymphocytes in draining LNs of kCYC$^{+/-}$ mice during the sensitization phase of CHS

We next examined the proliferation of lymphocytes in draining LNs. There were almost no gross and histological differences in the thymus, spleen, and peripheral LNs between WT and kCYC$^{+/-}$ mice without any stimulus (Sugaya *et al.*, 2005). Inguinal LNs were harvested before and after sensitization with 0.5% DNFB. The total numbers of draining LN cells increased after sensitization in both WT and kCYC$^{+/-}$ mice, although the increase was less remarkable in the latter (Figure 3a). The numbers of CD4^{+} T cells, CD8^{+} T cells, and B cells were also examined. Each cell type increased in number following sensitization in both types of mice (Figure 3a). Increases, however, were less remarkable in kCYC$^{+/-}$ mice when compared with WT mice, especially for CD8^{+} T cells and B cells. We next examined correlations between frequencies of DCs and those of CD4^{+} T cells, CD8^{+} T cells, and B cells in the draining LNs. As expected, frequencies of DCs strongly correlated with those of CD8^{+} T cells and B cells (Figure 3b), both of which are reported to be involved in CHS responses (Kehren *et al.*, 1999; Wang *et al.*, 2000; Larsen *et al.*, 2007; Watanabe *et al.*, 2007). On the other hand, frequencies of CD4^{+} T cells negatively correlated with those of DCs, which might reflect the relative increase of other cell populations. Interestingly, MHC class II expression on B cells significantly correlated with the numbers of B cells in draining LNs, suggesting activation of B cells in the draining LNs during the sensitization phase of CHS responses (Figure 3b).

Impaired CHS responses in kCYC$^{+/-}$ mice after removal of sensitized ear

Some topically applied antigens can be carried along by lymphatic flow and not be cell associated, and then picked up by resident DCs or B cells within draining LNs (Allenspach *et al.*, 2008; Lee *et al.*, 2009). We next examined the effects of mechanical blockade of antigen drainage to LNs on CHS responses. Sensitized ears were removed 1, 24, or 48 hours after application of 0.5% DNFB. CHS responses were completely abrogated when sensitized ears were removed 1 hour after painting in both WT and kCYC$^{+/-}$ mice (Figure 4a and b). When ears were removed 24 hours after sensitization, WT mice showed CHS responses comparable

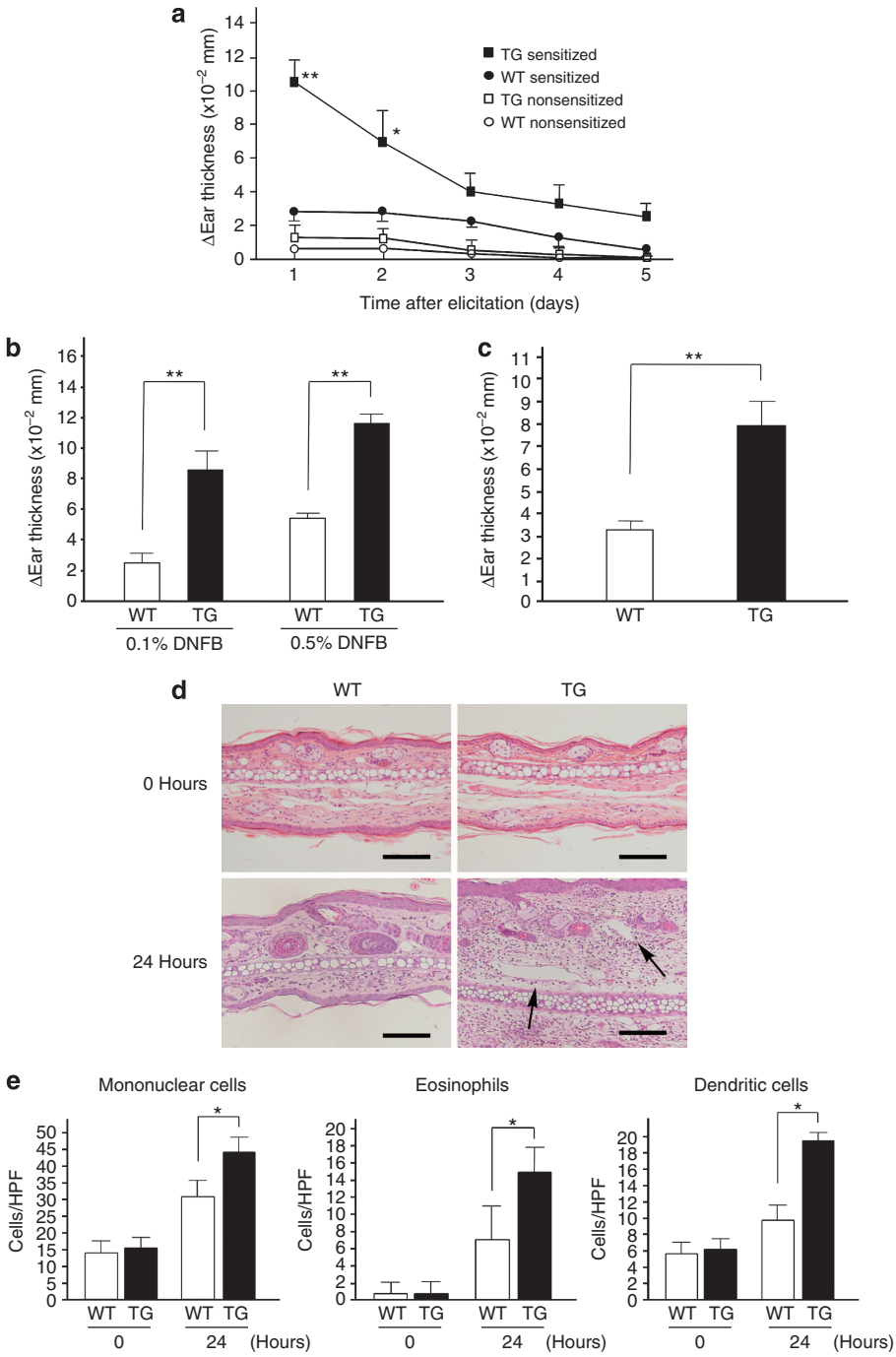

Figure 1. Augmented contact hypersensitivity (CHS) responses in *k-cyclin* transgenic (kCYC$^{+/-}$) mice. (**a**) Mice sensitized with 0.5% DNFB or nonimmunized mice were challenged with 0.25% DNFB. TG, transgenic; WT, wild type. (**b**) Mice were sensitized with 0.5 or 0.1% DNFB. Ear thickness was measured 24 hours after challenge. (**c**) Mice were sensitized with 0.5% FITC; $n = 10$ for each condition. *$P < 0.05$; **$P < 0.01$. (**d**) Hematoxylin and eosin (H&E) staining of sections from ears of wild-type (WT) and kCYC$^{+/-}$ mice before (0 hours) and 24 hours (24 hours) after elicitation (scale bar = 100 μm). Prominent dermal edema and dilated vessels (arrows) in the ear from kCYC$^{+/-}$ mice. Representative pictures from 10 mice per group. (**e**) The numbers of mononuclear cells, eosinophils, and dermal major histocompatibility complex (MHC) class II^{+} dendritic cells (DCs) per × 400 high-power fields (HPFs; $n = 5$). *$P < 0.05$.

to mice whose ears were not removed, whereas kCYC$^{+/-}$ mice showed almost no CHS responses. These results suggest that drainage of adequate antigen to induce normal CHS responses, either in free form or within migratory DCs, occurs within 24 hours in WT mice, as previously described (Turk and Stone, 1963), whereas this time range is not long enough to induce CHS in kCYC$^{+/-}$ mice with severe lymphatic dysfunction. In contrast, both WT and kCYC$^{+/-}$ mice whose ears were removed 48 hours after sensitization showed similar CHS responses as those without removal of

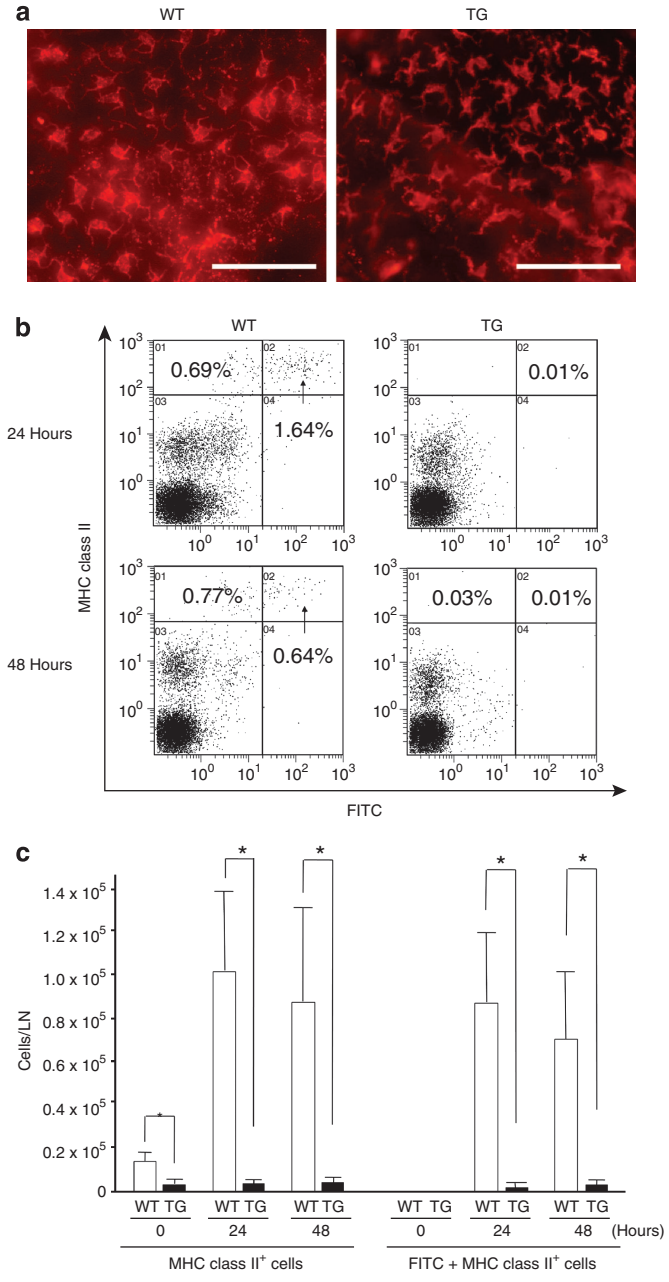

Figure 2. Impaired migration of skin-derived dendritic cells (DCs) into draining lymph nodes (LNs) of *k-cyclin* transgenic (kCYC$^{+/-}$) mice. (**a**) Epidermal sheets were stained with phycoerythrin (PE)-conjugated anti-I-A/I-E mAb. Representative pictures from four mice per group (scale bar = 100 μm). TG, transgenic; WT, wild type. (**b**, **c**) Inguinal LN cells were harvested 24 or 48 hours after applying 0.5% FITC on shaved abdomen ($n = 5$). (**b**) Representative data plots by flow cytometry are shown. (**c**) The numbers of major histocompatibility complex (MHC) class II$^+$ cells and FITC$^+$ MHC class II$^+$ cells were examined at the indicated times. *$P < 0.05$.

sensitized ears (data not shown). Quantitative analysis of infiltrating cells, including mononuclear cells, eosinophils, and neutrophils, showed similar results with skin thickness measurements (Figure 4c). kCYC$^{+/-}$ mice showed almost the same response as WT mice when sensitized ears were not removed, which was quite different from the ear thickness model (Figure 1). This prompted us to further investigate whether augmented ear swelling was from enhanced immunization or solely from impaired drainage following elicitation.

Impaired lymphatic drainage in the elicitation phase of CHS enhances apparent inflammation in kCYC$^{+/-}$ mice

Severe lymphatic dysfunction induced impaired migration of skin DCs, as well as free antigen drainage to regional LNs, which could not explain augmented ear swelling in kCYC$^{+/-}$ mice. Therefore, we adoptively transferred sensitized lymphocytes and challenged transplanted mice to investigate components in the elicitation phase of CHS. WT mice transferred with sensitized lymphocytes from either WT or kCYC$^{+/-}$ mice showed minimal ear swelling (Figure 5a). In contrast, kCYC$^{+/-}$ mice transferred with sensitized lymphocytes either from WT or kCYC$^{+/-}$ mice showed enhanced ear swelling. No ear swelling was detected when sensitized lymphocytes were not transferred to mice. In addition, kCYC$^{+/-}$ mice showed stronger irritant responses compared with WT mice following application of croton oil (Figure 5b), suggesting that impaired drainage was critically important for augmented ear swelling in kCYC$^{+/-}$ mice.

We next examined antigen-specific IFN-γ production and proliferative T-cell responses. IFN-γ enzyme-linked immunospot assay revealed more IFN-γ-producing cells in inguinal LNs in WT mice compared with kCYC$^{+/-}$ mice (Figure 5c). Almost no spots were detected in cell suspensions from unsensitized mice or from cells not restimulated with antigen. Antigen-specific proliferative responses were also much higher in WT mice compared with kCYC$^{+/-}$ mice (Figure 5d), suggesting decreased immunization in the setting of lymphatic dysfunction. Moreover, we assessed IFN-γ, tumor necrosis factor-α, CXCL9 (chemokine (C-X-C motif) ligand 9), and CXCL10 (chemokine (C-X-C motif) ligand 10) mRNA expression in challenged ears, all of which were reported to be strongly associated with CHS responses (Goebeler *et al.*, 2001; Ogawa *et al.*, 2010). Surprisingly, ears of kCYC$^{+/-}$ mice 24 hours after challenge contained significantly lower amounts of IFN-γ, tumor necrosis factor, CXCL9, and CXCL10 mRNAs compared with WT mice (Figure 5e). These results strongly suggested that augmented ear swelling of kCYC$^{+/-}$ mice was mainly due to retention of infiltrating cells and fluid within inflamed tissue.

DISCUSSION

In this study, we have demonstrated that transgenic mice with severe lymphatic dysfunction have enhanced ear swelling. Although migration of skin-derived DCs and establishment of antigen-specific T cells were impaired in the sensitization phase, defects in drainage of accumulated inflammatory cells and fluid in the elicitation phase dominated and resulted in augmented ear swelling overall. The results of this study provide insight into the immunopathological basis of contact dermatitis and venous stasis dermatitis commonly observed in humans with peripheral lymphedema of the lower legs.

We were surprised to see that ear swelling was significantly augmented in kCYC$^{+/-}$ mice compared with WT mice

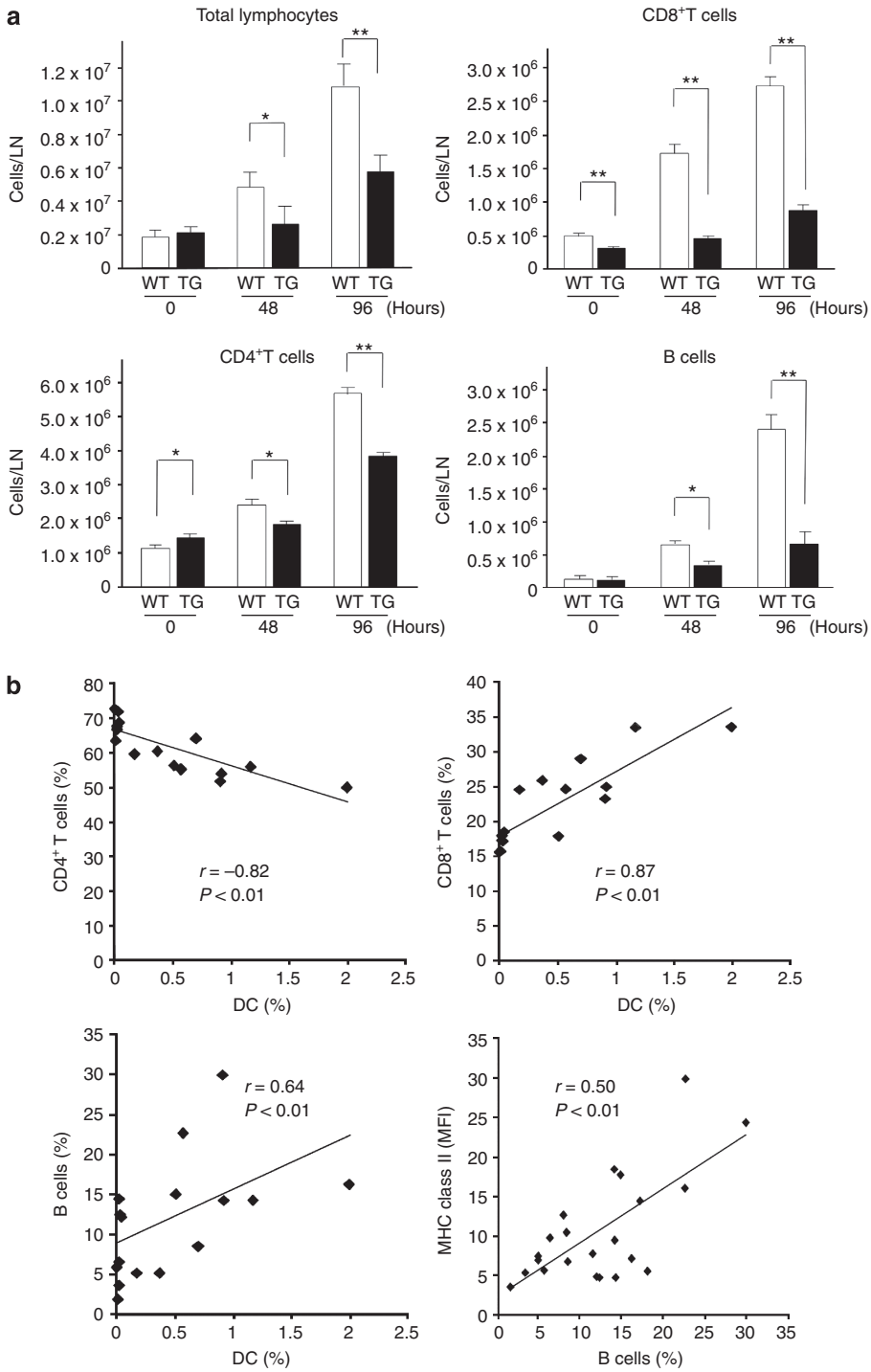

Figure 3. Impaired proliferation of lymphocytes in the draining lymph nodes (LNs) of *k-cyclin* transgenic (kCYC$^{+/-}$) mice. Inguinal LN cells were harvested 0, 48, or 96 hours after applying 0.5% DNFB on shaved abdominal skin. The cells were stained for FITC-conjugated anti-CD8, FITC-conjugated anti-B220, phycoerythrin (PE)-conjugated anti-I-A/I-E, PE-conjugated anti-CD11c, and PE-conjugated anti-CD4 mAbs and were analyzed by flow cytometry ($n = 5$). (**a**) Time course of cell numbers of each population in the draining LNs. *$P < 0.05$; **$P < 0.01$. (**b**) Correlations between frequencies of dendritic cells (DCs) and those of CD4$^+$ T cells, CD8$^+$ T cells, and B cells in the draining LNs, and correlation between frequencies of B cells and major histocompatibility complex (MHC) class II expression on B cells. MFI, mean fluorescence intensity.

(Figure 1). Migration of antigen-bearing DCs to the regional LNs is believed to be important for CHS responses, especially in the sensitization phase. Therefore, augmented ear swelling in kCYC$^{+/-}$ mice prompted us to investigate whether DCs could migrate to draining LNs regardless of impaired lymphatic flow. Migratory DCs move actively via interactions between CCL21 (chemokine (C-C motif) ligand 21) and CCR7 (chemokine (C-C motif) receptor 7) (Saeki *et al.*, 1999;

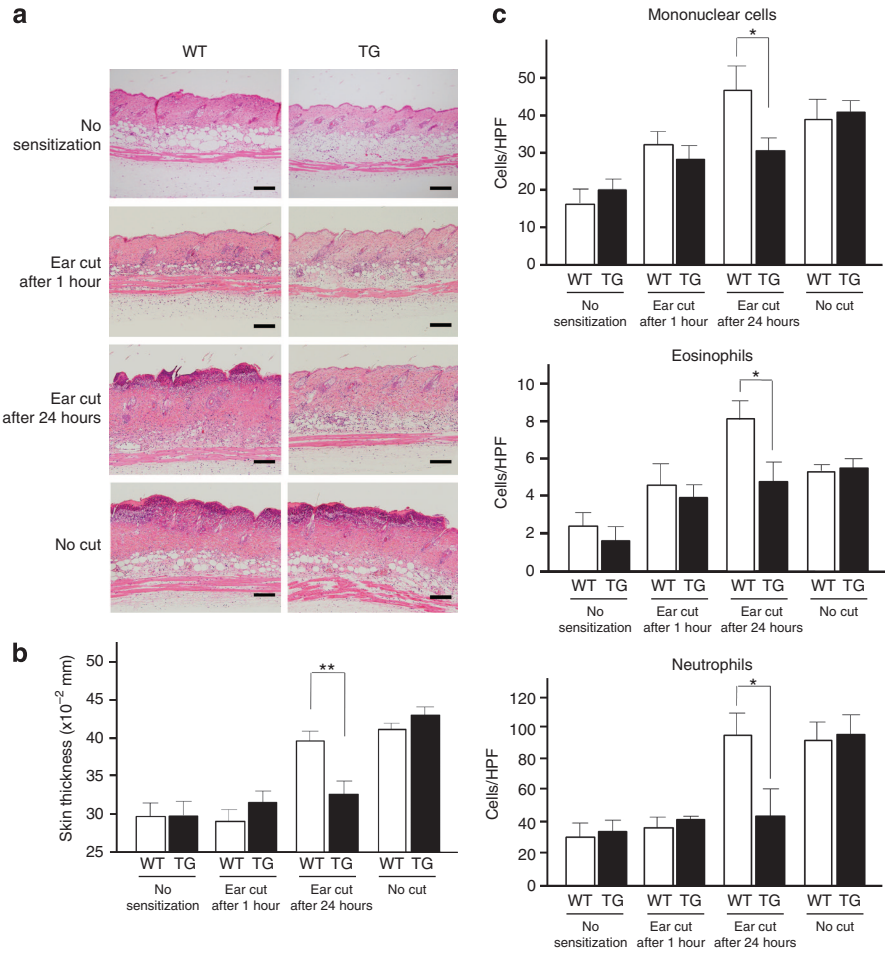

Figure 4. Impaired contact hypersensitivity (CHS) responses in *k-cyclin* transgenic (kCYC$^{+/-}$) mice after removal of sensitized ears. (**a**) Sensitized ears were removed 1 or 24 hours after the application of 0.5% DNFB. For some mice, ears were not removed as positive controls (no cut). Nonsensitized mice were used as negative controls (no sensitization). CHS responses were elicited by applying 20 µl of 0.25% DNFB onto shaved back skin. Sections from back skin 24 hours after challenge were stained for hematoxylin and eosin (H&E). Representative pictures from five mice per group (scale bar = 100 µm). TG, transgenic; WT, wild type. (**b**) Skin thickness was measured for each condition ($n = 5$). $^{**}P < 0.01$. (**c**) The numbers of mononuclear cells, eosinophils, and neutrophils per ×400 high-power fields (HPFs; $n = 5$). $^{*}P < 0.05$.

Ohl *et al.*, 2004). Lymphatic retention in our transgenic mice almost completely blocked migration of skin DCs to draining LNs (Figure 2). Abrogation of a chemotactic gradient might explain the impaired DC migration.

Once antigen-bearing DCs reach draining LNs, proliferation of antigen-specific lymphocytes commences. The numbers of lymphocytes in draining LNs increased 3 to 5 days following topical antigen exposure (Macatonia *et al.*, 1987; Tomei *et al.*, 2009). The transgenic mice in this study also showed increases in draining LN lymphocytes at similar time points, although the degree was less remarkable than in WT mice (Figure 3a). This suggests that immune responses, although less strong, occur within LNs of kCYC$^{+/-}$ mice after sensitization. Proliferation of CD8$^+$ T cells and B cells was impaired in those mice (Figure 3a). CHS responses are largely mediated by CD8$^+$ T cells (Kehren *et al.*, 1999; Wang *et al.*, 2000), but B cells are also activated and involved in CHS responses (Larsen *et al.*, 2007; Watanabe *et al.*, 2007). Activation of CD8$^+$ T cells and B cells seems to be mainly mediated by migratory DCs (Macatonia *et al.*, 1987). Consistently, frequencies of DCs strongly correlated with those of CD8$^+$ T cells and B cells (Figure 3b). As very few numbers of migratory DCs in LNs of kCYC$^{+/-}$ mice were observed before 96 hours after sensitization (Figure 2 and data not shown), this may explain why we observed impaired numbers of CD8$^+$ T cells and B cells. On the other hand, proliferation of CD4$^+$ T cells was not so impaired in transgenic mice (Figure 3a), suggesting that these cells may be activated during CHS in a DC-independent manner. MHC class II expression on B cells increased in WT mice, suggesting activation of B cells during CHS, whereas MHC class II expression on B cells remained low in kCYC$^{+/-}$ mice. Interestingly, MHC class II expression on B cells significantly correlated with the numbers of B cells in draining LNs (Figure 3b).

Migratory DCs are mainly composed of epidermal Langerhans cells and dermal DCs (Ohl *et al.*, 2004). The roles of Langerhans cells in CHS responses are now

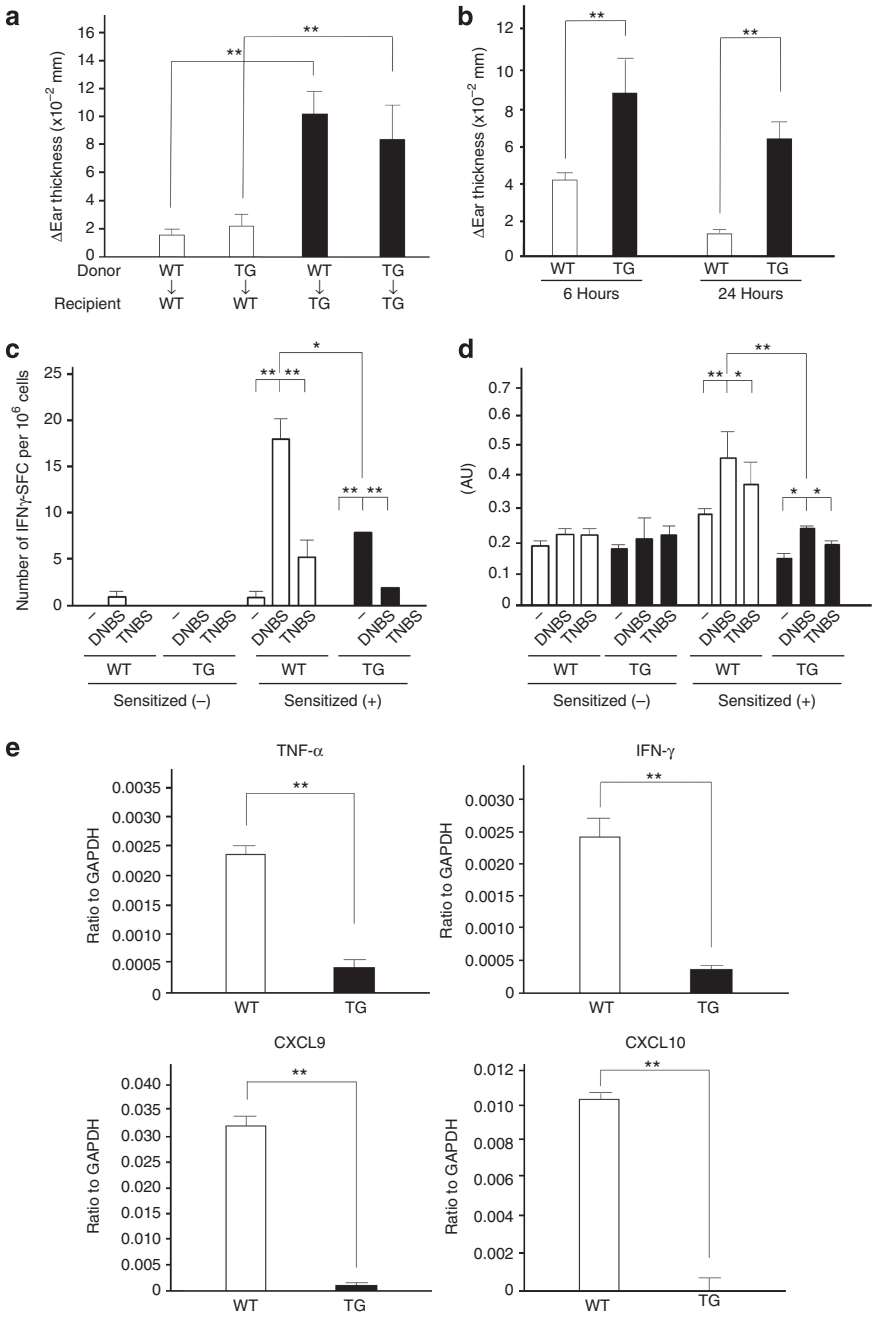

Figure 5. The elicitation phase is critical for augmenting contact hypersensitivity (CHS) responses in *k-cyclin* transgenic (kCYC$^{+/-}$) mice. (**a**) Inguinal lymph node (LN) cells from DNFB-sensitized mice were adoptively transferred intravenously. Recipient mice were elicited with 0.25% DNFB 24 hours after the transfer. Ear swelling responses were measured 24 hours after challenge. (**b**) Croton oil was applied to the mouse ear. After 6 and 24 hours, changes in ear thickness were measured ($n = 10$). (**c**) Enzyme-linked immunospot (ELISPOT) assay using inguinal LN cells from sensitized and nonsensitized mice. (**d**) BrdU proliferation assay using inguinal LN cells from sensitized and nonsensitized mice. (**e**) Ears challenged with DNFB were harvested and RNA was obtained. Quantitative reverse transcription-PCR (RT-PCR) was performed for IFN-γ, tumor necrosis factor-α (TNF-α), CXCL9 (chemokine (C-X-C motif) ligand 9), and CXCL10 (chemokine (C-X-C motif) ligand 10). *$P<0.05$; **$P<0.01$. One representative result from two independent experiments with triplicates (**a**, **c–e**). AU, arbitrary unit; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SFC, spot-forming cell; TG, transgenic; TNBS, trinitrobenzene sulfonic acid; WT, wild type.

controversial (Bennett *et al.*, 2005; Kaplan *et al.*, 2005; Teoh *et al.*, 2009). Furthermore, it has been revealed that some antigens are presented by DCs residing in LNs (Allenspach *et al.*, 2008; Lee *et al.*, 2009). Therefore, very few antigen-bearing DCs within LNs of kCYC$^{+/-}$ mice prompted us to investigate the role of free antigen directly carried into the draining LNs. Results revealed that 24 hours was long enough for the drainage of antigens, either in free form or within migratory DCs, in WT mice, whereas 48 hours was necessary for kCYC$^{+/-}$ mice (Figure 4). These results were comparable

to our previous study in which we used injected dye and lymphangiograms to assess lymphatic flow (Sugaya *et al.*, 2005). Free antigens can travel much faster than antigen-bearing DCs. It was reported that fluorescent DCs in the draining LNs were detectable 30 minutes after skin painting with FITC, which appears to be too early for skin DCs to reach LNs (Saeki *et al.*, 1999). Fluorescent B cells were also detected 1 day following sensitization. Taken together, resident DCs or B cells in LNs may take up cell-free antigen flowing through lymphatic vessels and may be involved in establishing memory T cells.

Consistent with impaired DC migration and LN lymphocyte proliferation, antigen-specific IFN-γ production and proliferative responses were decreased in kCYC$^{+/-}$ mice (Figure 5c and d). Cytokine mRNA expression in the challenged ears from transgenic mice were also significantly decreased (Figure 5e). It was surprising to see a discrepancy between ear thickness and cytokine expression. Our results reveal that ear thickness does not necessarily reflect the degree of immune reaction within tissue. Irritant dermatitis induced by croton oil, which does not need prior sensitization, represents a nonspecific response to foreign antigen. Enhanced irritant dermatitis in kCYC$^{+/-}$ mice points to the importance of lymphatic vessels to clear fluid and infiltrating cells from inflamed skin. Not only do lymphocytes and DCs use lymphatics, but erythrocytes are collected through lymphatic vessels as well (Kissenpfennig *et al.*, 2005). We previously showed that erythrocytes were detected in dilated lymphatic vessels in tagged ears of kCYC$^{+/-}$ mice (Sugaya *et al.*, 2005). During inflammation, tissue fluid drainage can be increased by $\geqslant$10-fold (Flessner *et al.*, 1983; Fischer *et al.*, 1996). Impaired lymphatic system in kCYC$^{+/-}$ mice, which do not show clinical symptoms of skin disease in the absence of skin inflammation, cannot adequately manage the increase in tissue fluid and cells during inflammation. Defects in the drainage of accumulated inflammatory cells and fluid in the elicitation phase of CHS leads to augmented ear swelling, despite impaired DC migration during the sensitization phase of CHS. Thus, our findings point to a dominant role for lymphatic drainage in clearing inflammatory cells from tissue and in resolving tissue inflammation following the onset of cutaneous inflammation. These findings are also clinically relevant in that they provide mechanistic insight into the problem of allergic contact dermatitis and venous stasis dermatitis, which frequently occur in the lower legs of individuals with severe lymphedema.

Although VEGFR-3 is mainly expressed on lymphatic endothelial cells, other cell types such as corneal DCs, murine macrophages, and B-cell chronic lymphocytic leukemia cells can express VEGFR-3 (Hamrah *et al.*, 2003; Bairey *et al.*, 2004; Stepanova *et al.*, 2007). When we previously analyzed transgene expression, kCYC mRNA signals localized to karyomegalic lymphatic endothelial cells lining vessels positive for VEGFR-3 and podoplanin (Sugaya *et al.*, 2005). Expression of the kCYC transgene in different cell types, however, cannot be completely ruled out, and thus may have a role in the functional changes observed in our experiments. Other mouse lymphedema models or studies using human tissues or cells would be necessary to address this issue.

MATERIALS AND METHODS

Mice

FVB/N mice were purchased from Clea Japan (Tokyo, Japan). kCYC$^{+/-}$ mice were generated as previously described (Sugaya *et al.*, 2005). All mice were free of pathogenic bacteria and viruses. All experiments were conducted using mice between 6 and 14 weeks of age. All studies and procedures were approved by the Animal Committee of National Center for Global Health and Medicine.

Sensitization and elicitation of CHS

CHS responses were induced either with DNFB or FITC, as previously described (Watanabe *et al.*, 2007). A volume of 50 µl of 0.5 or 0.1% DNFB was painted onto shaved abdominal skin on day 0, and CHS was elicited by applying 0.25% DNFB on the left ear on day 5. CHS responses to FITC were induced by applying 0.5% FITC to shaved abdominal skin. After 5 days, CHS reactions were elicited by applying FITC solution. For all CHS experiments, baseline ear thickness was determined with a spring-loaded caliper. 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 three times by a researcher who was blind to the results and the mean of these values was used. Croton oil was used to elicit irritant contact dermatitis. A volume of 15 µl of 2.0% croton oil was painted on the left ear. After 6 and 24 hours, the change in ear thickness from baseline was measured as described. DNFB, FITC, and croton oil were purchased from Sigma-Aldrich (St Louis, MO).

Removal of sensitized ear

To evaluate the effects of delay in antigen draining to LN during CHS responses, 20 µl of 0.5% DNFB was applied on the left ear on day 0. Ears were removed 1, 24, or 48 hours after the application. CHS responses were elicited by applying 0.25% DNFB on the shaved back skin on day 5. The back skin was removed 24 hours after the challenge and was assessed histologically. Skin thickness was histologically measured.

Histological examination

Ear or back skin samples were fixed in 4% formalin and embedded in paraffin. Sections, 4 µm thick, were cut and stained with hematoxylin and eosin. Skin thickness was histologically measured. The numbers of mononuclear cells and eosinophils were counted in 10 random grids under magnification of ×400 high-power fields and averaged. In some experiments, ear skin samples were snap-frozen, cut into 5-µm-thick cryostat sections, and fixed in acetone. These sections were then stained with phycoerythrin (PE)-conjugated anti-I-A/I-E mAb. The numbers of dermal MHC class II^{+} DCs were counted. Each section was examined independently by two investigators in a blinded manner.

Quantitative reverse transcription-PCR to assess cytokine production in the challenged ears

The ears of WT and kCYC$^{+/-}$ mice were challenged with DNFB as described above. After 24 hours, the ears were harvested and RNA

was obtained using the RNeasy Fibrous Tissue Mini Kit (QIAGEN, Valencia, CA). Complementary DNA was synthesized using TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA). Quantitative reverse transcription-PCR was performed as described previously (Sugaya *et al.*, 2006). Primers for mouse IFN-γ, tumor necrosis factor, CXCL9, CXCL10, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were as follows: IFN-γ forward, 5′-AGCAACAGCAAGGCGAAAA-3′ and reverse, 5′-CTGGACCTGT GGGTTGTTGA-3′; tumor necrosis factor forward, 5′-CCACCACGCT CTTCTGTCTAC-3′ and reverse, 5′-AGGGTCTGGGCCATAGAAC T-3′; CXCL9 forward, 5′-TGGGCATCATCTTCCTGGAG-3′ and reverse, 5′-CCGGATCTAGGCAGGTTTGA-3′; CXCL10 forward, 5′-CCCACGTGTTGAGATCATTG-3′ and reverse, 5′-CACTGGGTAA ACGGGAGTGA-3′; GAPDH forward, 5′-CGTGTTCCTACCCCCAAT GT-3′ and reverse, 5′-TGTCATCATACTTGGCAGGTTTCT-3′.

Flow cytometry

Inguinal LNs were harvested at the described time after sensitization, and cell suspensions were prepared by digesting tissues with 1 mg ml^{-1} collagenase D (Sigma-Aldrich) and 0.2 mg ml^{-1} DNase (Sigma-Aldrich). Single-cell suspensions were stained for two-color immunofluorescence analysis at 4 °C using FITC-conjugated anti-CD8, FITC-conjugated anti-B220, PE-conjugated anti-I-A/I-E, PE-conjugated anti-CD11c, and PE-conjugated anti-CD4 mAbs (BD PharMingen, San Diego, CA) for 20 minutes. Labeled cells were analyzed on an EPICS XL flow cytometer (Beckman Coulter, Fullerton, CA) with fluorescence intensity shown on a 4-decade log scale. Positive and negative populations of cells were determined using isotype-matched Abs (Southern Biotechnology, Birmingham, AL) as controls for background staining. Mean fluorescence intensity for MHC class II on B220$^+$ cells (B cells) was determined for each experiment.

Adoptive transfer of sensitized LN cells

Donor mice were sensitized with 0.5% DNFB on day 0 as described above. On day 5, inguinal LN cells were harvested and a mixture of 2–4 × 10^6 cells in 200 μl of phosphate-buffered saline was adoptively transferred intravenously. After 24 hours, mice were elicited with 0.25% DNFB and ear swelling responses were measured after 24 hours.

IFN-γ enzyme-linked immunospot assay

Inguinal LNs were harvested 5 days after DNFB sensitization. Cell suspensions were restimulated *in vitro* by overnight culture with mitomycin C-treated syngeneic spleen cells (10^6 per well) from naive mice in complete RPMI medium supplemented with 10% fetal calf serum and containing a final concentration of 0.4 mM DNBS. Control cultures included cells cultured overnight in medium supplemented with 0.2 mM of the irrelevant hapten trinitrobenzene sulfonic acid, or in medium alone. The number of IFN-γ-producing cells was determined using an enzyme-linked immunospot assay kit (R&D systems, Minneapolis, MN). The number of IFN-γ spot-forming cells present in each well was counted using a microscope, and the results were expressed as IFN-γ spot-forming cells per 10^6 cells.

Hapten-specific T-cell proliferation *in vitro*

Inguinal LNs were harvested 5 days after DNFB sensitization. Cell suspensions were cocultured for 3 days with mitomycin C-treated syngeneic spleen cells (10^6 per well) from naive mice, which had been previously incubated for 20 minutes at 37 °C with 4 mM DNBS, 2 mM trinitrobenzene sulfonic acid, or medium only and washed in complete medium before use. Cells were stained with BrdU for 16 hours and reacted with anti-BrdU Ab peroxidase conjugate, followed by peroxidase substrate using Cell Proliferation ELISA, BrdU (Roche Applied Science, Basel, Switzerland). Sulfuric acid was added to the solution to terminate enzyme activity. Optical densities were measured at 450 nm using a 550 microplate reader (Bio-Rad Laboratories, Hercules, CA).

Statistics

All data are shown as mean values + SEM. Statistical analysis between two groups was performed using the Mann–Whitney *U*-test. Correlation coefficients were determined using Spearman's rank correlation test. The *P*-values of <0.05 were considered statistically significant.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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