

# Cutaneous Hypersensitivities to Hapten Are Controlled by IFN- $\gamma$ -Upregulated Keratinocyte Th1 Chemokines and IFN- $\gamma$ -Downregulated Langerhans Cell Th2 Chemokines

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There are immediate, late-phase, and delayed-type reactions to exogenous agents. In IFN- $\gamma$ -knockout (IFN- $\gamma^{-/-}$ ) and wild-type B6 mice, we examined the response to picryl chloride (PCI) for assessing delayed-type reactions, and the responses to repeatedly challenged FITC for immediate and late-phase reactions. The delayed-type hypersensitivity was depressed in IFN- $\gamma^{-/-}$  mice, and the immediate and late-phase reactions were enhanced in IFN- $\gamma^{-/-}$  mice. As skin-infiltrating lymphocytes were scarce at the PCI-challenged site of IFN- $\gamma^{-/-}$  mice, we investigated chemokine production by keratinocytes and Langerhans cells (LCs). A real-time PCR analysis demonstrated that Th1 chemokines (CXCL9 and CXCL10) and Th2 chemokines (CCL17 and CCL22) were derived mainly from keratinocytes and LCs, respectively. Challenge with PCI or FITC augmented keratinocyte expression of Th1 chemokines in wild-type but not in IFN- $\gamma^{-/-}$  mice, and Th2 chemokine production by LCs was induced by repeated FITC in IFN- $\gamma^{-/-}$  mice. Finally, transfer of carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled draining lymph node cells from hapten-sensitized B6 mice or lymph node cells from sensitized green fluorescent protein (GFP) mice to naive IFN- $\gamma^{-/-}$  mice revealed less infiltration of CFSE<sup>+</sup> or GFP<sup>+</sup> lymphocytes at the challenged site. Our study suggests that one of the crucial actions of IFN- $\gamma$  is upregulation of keratinocyte production of Th1 chemokines and downregulation of LC production of Th2 chemokines.

*Journal of Investigative Dermatology* (2008) **128**, 1719–1727; doi:10.1038/jid.2008.5; published online 31 January 2008

## INTRODUCTION

IFN- $\gamma$  is one of the critical cytokines involved in cutaneous hypersensitivity responses as well as in systemic immune reactions. First, as a representative Th1 cytokine, IFN- $\gamma$  supports CD8<sup>+</sup> effector T cells that evoke contact hypersensitivity (CHS) response (Bour *et al.*, 1995; Xu *et al.*, 1996), graft-versus-host reaction (Blazar *et al.*, 1998), and tumor immunity (Seo and Tokura, 1999). Second, IFN- $\gamma$  upregulates the immunological functions of keratinocytes, including expression of CD54 (Griffiths *et al.*, 1990), production of proinflammatory cytokines such as IL-1 $\alpha$  and tumor necrosis factor (TNF)- $\alpha$  (Pastore *et al.*, 1998), production of chemo-

kines such as IL-8, CCL5/RANTES (regulated upon activation, normal T-cell expressed and secreted) (Li *et al.*, 1996), and Th1- and Th2-associated chemokines (Sebastiani *et al.*, 2002). Finally, IFN- $\gamma$  modulates the various functions of Langerhans cells (LCs), including expression of major histocompatibility complex class II and co-stimulatory molecules (Salgado *et al.*, 1999) and production of cytokines and chemokines (Matsue *et al.*, 1992).

Although knowledge regarding the *in vitro* effects of IFN- $\gamma$  on skin immunocompetent cells has been thus accumulating, one can realize that the *in vivo* actions of IFN- $\gamma$  have not extensively been studied. For example, as a Th1 cytokine, systemic IFN- $\gamma$  skews Th balance to Th1, and the treatment of Th2 disorders is improved by IFN- $\gamma$ . However, patients with atopic dermatitis are not necessarily alleviated by IFN- $\gamma$  (Stevens *et al.*, 1998). In another example, IFN- $\gamma$  promotes the *in vitro* production of Th2 chemokines by keratinocytes (Sebastiani *et al.*, 2002), but it remains unknown as to whether this seemingly ambivalent phenomenon really occurs in *in vivo* settings. To resolve these issues, *in vivo* studies using IFN- $\gamma$ -deficient mice are required.

Contact hypersensitivity belongs to the delayed-type hypersensitivity (DTH), peaks at 24–48 hours after challenge, and involves Th1/Tc1 cells (Akiba *et al.*, 2002), cutaneous dendritic cells (epidermal LCs and dermal dendritic cells)

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Abbreviations: B6, C57BL/6J; CFSE, carboxyfluorescein diacetate succinimidyl ester; CHS, contact hypersensitivity; C<sub>t</sub>, cycle threshold; DTH, delayed-type hypersensitivity; EC, epidermal cell; GFP, green fluorescent protein; IFN- $\gamma^{-/-}$ , IFN- $\gamma$ -knockout; LC, Langerhans cell; PCI, picryl chloride

Received 15 August 2007; revised 11 December 2007; accepted 12 December 2007; published online 31 January 2008

serving as antigen-presenting cells (Kissenpfennig and Malissen, 2006), and keratinocytes producing IL-1 $\alpha$ , TNF- $\alpha$ , and GM-CSF (Heufler *et al.*, 1998) stimulatory for LCs (Sugita *et al.*, 2007). However, when a given contactant is prone to stimulate Th2 cells, certain responses earlier than DTH take place and include immediate and late-phase reactions. The immediate type occurs at 15 minutes to 1 hour and is induced by IgE and mast cells (Kitagaki *et al.*, 1995), and the late-phase reaction peaks at 4–8 hours and is mediated by Th2 cells and eosinophils (Dearman and Kimber, 2000; Ying *et al.*, 2002).

Chemokines derived from epidermal cells (ECs) are one of the recent topics in CHS responses. Keratinocytes secrete Th1 chemokines, CXCL9/Mig and CXCL10/IP-10, and Th2 chemokines, CCL17/TARC and CCL22/MDC (Sebastiani *et al.*, 2002). IFN- $\gamma$  stimulates keratinocytes to produce both Th1 and Th2 chemokines (Kakinuma *et al.*, 2002; Sebastiani *et al.*, 2002). The stimulatory ability of IFN- $\gamma$  for Th1 chemokines appears to be reasonable, as a Th1-polarized local response may be induced by IFN- $\gamma$ . However, its ability to produce Th2 chemokines needs some sophisticated idea to harmoniously construct sequential events. In contrast, LCs are capable of producing Th2 and Th1 chemokines *in vitro*, including CCL17, CCL22, CXCL10, CXCL9, and CXCL11/I-TAC with Th2 chemokine dominancy. IFN- $\gamma$  is mostly suppressive for the LC expression of Th2 chemokines, CCL17 and CCL22 (Fujita *et al.*, 2005). Thus, IFN- $\gamma$  can modulate both keratinocytes and LCs in their chemokine production and in the resultant occurrence of CHS.

In this study, we addressed the role of IFN- $\gamma$  for the immediate, late-phase, and delayed-type cutaneous hypersensitivities by using IFN- $\gamma$ -knockout (IFN- $\gamma^{-/-}$ ) mice. As our preliminary study showed the extent of skin infiltration of lymphocytes was different between the wild-type and IFN- $\gamma^{-/-}$  mice, we focused on the effects of IFN- $\gamma$  on the chemokine production by keratinocytes and LCs. Results suggest that Th1 and Th2 chemokines are produced mainly by keratinocytes and LCs, respectively, and IFN- $\gamma$  upmodulates the production of Th1 chemokines by keratinocytes but downmodulates the production of Th2 chemokines by LCs, leading to the

reduction of cutaneous DTH response and the enhancement of the early responses.

## RESULTS

### Reduced CHS response to 2,4,6-trinitrochlorobenzene (picryl chloride) in IFN- $\gamma^{-/-}$ mice

Initially, we tested the degree of ordinary CHS response in IFN- $\gamma^{-/-}$  mice along with wild-type C57BL/6J (B6) mice. Mice were sensitized and challenged with picryl chloride (PCI), and their ear swelling responses were measured 24 hours after challenge. A significant degree of ear swelling response was observed in B6 but not in IFN- $\gamma^{-/-}$  mice, as compared to the negative control mice challenged without sensitization (Figure 1a). CHS responses to PCI were monitored at 1, 4, and 24 hours after challenge. IFN- $\gamma^{-/-}$  mice did not exhibit a substantial swelling response throughout the time course (Figure 1b). The ear swelling response of B6 mice was reduced at 48 hours ( $7.0 \times 10^{-3}$  cm) compared to that at 24 hours. No significant difference was observed in the ear swelling of IFN- $\gamma^{-/-}$  mice between 24 and 48 hours (data not shown).

Histologically, the intensity of inflammatory cell infiltrate and dermal edema at the PCI-challenged site was lower in IFN- $\gamma^{-/-}$  mice than in B6 mice (Figure 2a). When CD3<sup>+</sup> T cells in the dermis and subcutaneous tissue were enumerated, T cells scarcely infiltrated in IFN- $\gamma^{-/-}$  mice (Figure 2b). Thus, IFN- $\gamma^{-/-}$  mice lacked a capacity to develop cutaneous DTH, and this inability was associated with the absence of skin-infiltrating T cells.

### Elevated immediate and late-phase reactions to repeatedly challenged FITC in IFN- $\gamma^{-/-}$ mice

To evaluate whether IFN- $\gamma^{-/-}$  mice can develop early-phase cutaneous reactions, we used the repeated hapten challenge system, a known method to induce a Th2-mediated early skin response (Kitagaki *et al.*, 1997), with the use of Th2-skewing hapten FITC (Dearman and Kimber, 2000). FITC was painted one, three, or six times on the ears of B6 and IFN- $\gamma^{-/-}$  mice that were sensitized with this hapten. The single challenge with FITC did not elicit any ear swelling response at 1, 4, or

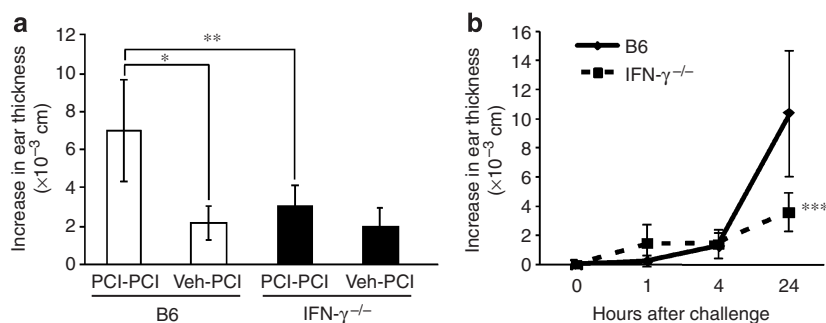


**Figure 1. CHS to PCI in B6 and IFN- $\gamma^{-/-}$  mice.** (a) B6 and IFN- $\gamma^{-/-}$  mice were sensitized with 5% PCI on the shaved abdomen and challenged with 0.5% PCI on the ears (PCI-PCI). Control mice were painted with vehicle alone and challenged with PCI (Veh-PCI). Ear thickness swelling was measured 24 hours later. (b) The time course of ear swelling responses was monitored after elicitation with PCI in B6 and IFN- $\gamma^{-/-}$  mice. Data are expressed as the mean  $\pm$  SD of six mice. \* $P=0.0016$ , \*\* $P=0.00029$ , and \*\*\* $P=0.0060$ .

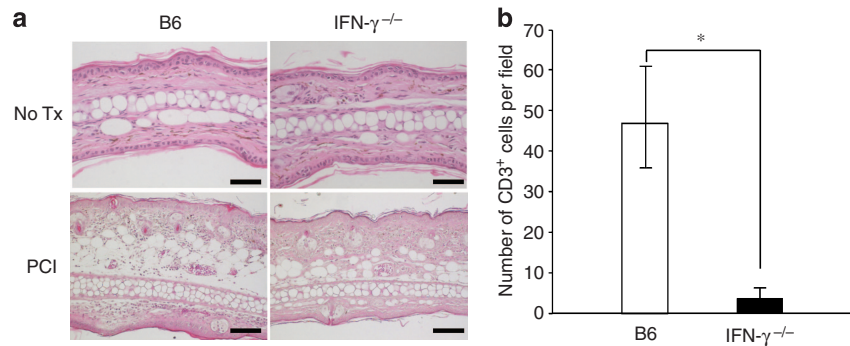


**Figure 2. Histology and numbers of CD3<sup>+</sup> T cells in PCI-challenged skin of B6 and IFN- $\gamma$ <sup>-/-</sup> mice.** (a) B6 and IFN- $\gamma$ <sup>-/-</sup> mice were sensitized with 5% PCI and challenged with 0.5% PCI. The challenged ears, along with no-treated ones (no Tx), were stained with hematoxylin and eosin. (b) Cryostat sections of the challenged ears were immunohistochemically stained with anti-CD3 mAb, and CD3<sup>+</sup> cells were enumerated in original magnification  $\times 500$  field. Data are expressed as the mean  $\pm$  SD of five mice. \* $P=0.00018$ . Bar = 80  $\mu$ m.

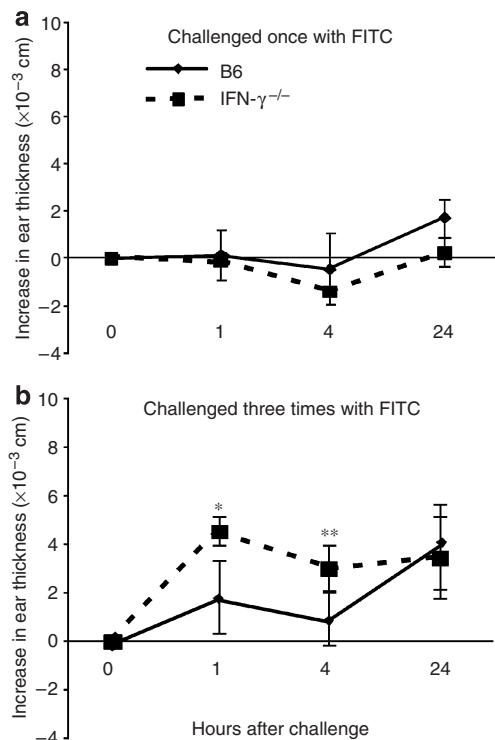


**Figure 3. Time course of ear swelling responses of B6 and IFN- $\gamma$ <sup>-/-</sup> mice receiving single-time or three time-elicitation with FITC.** B6 and IFN- $\gamma$ <sup>-/-</sup> mice were sensitized with 1% FITC on the abdomen and challenged with 0.5% FITC once or thrice (3- to 4-day intervals) on the ears. One, four, and 24 hours after the final challenge, the ear swelling responses were measured. Data are expressed as the mean  $\pm$  SD of five mice. \* $P=0.0048$ , \*\* $P=0.0060$ .

24 hours after challenge in either strain of mice (Figure 3a). When challenged three times, significantly higher swelling responses were found at 1 and 4 hours in IFN- $\gamma$ <sup>-/-</sup> mice than in B6 mice (Figure 3b). There was no difference between the two strains at 24 hours. The six-time challenge induced similar but slightly higher levels of these reactions (data not shown). The results suggested that IFN- $\gamma$ <sup>-/-</sup> mice are prone to develop immediate and late-phase cutaneous reactions instead of DTH, suggesting a Th2-skewed immunological state.

#### Augmentation of IL-4 expression by repeated FITC challenge in immune lymph nodes of IFN- $\gamma$ <sup>-/-</sup> mice

To confirm the preponderant expression of Th2 cytokines in IFN- $\gamma$ <sup>-/-</sup> mice, B6 and IFN- $\gamma$ <sup>-/-</sup> mice were sensitized with PCI or FITC, and challenged once with PCI or six times with FITC, respectively. Cervical lymph nodes were taken from these mice 24 hours after the last challenge, and IFN- $\gamma$  and IL-4 expressions were examined by real-time PCR. B6 mice had IFN- $\gamma$  expression at a higher level by single challenge with PCI than by repeated challenge with FITC, whereas IFN- $\gamma$ <sup>-/-</sup> mice naturally exhibited no IFN- $\gamma$  expression (Figure 4). As for IL-4, the repeated FITC challenge enhanced its expression in both strains of mice as compared to PCI challenge, but the level of enhancement by FITC was greatly higher in IFN- $\gamma$ <sup>-/-</sup> mice than in B6 mice. These data indicated preferential activation of Th2 cells in IFN- $\gamma$ <sup>-/-</sup> mice after repeated FITC challenge.

#### Infiltration of Th2 cells and presence of IgE and IL-4 at repeatedly FITC-challenged skin of IFN- $\gamma$ <sup>-/-</sup> mice

B6 and IFN- $\gamma$ <sup>-/-</sup> mice were sensitized and challenged six times with FITC, and their ears were histologically examined. Both types of mice exhibited an inflammatory infiltrate mainly in the dermis (Figure 5a). Some lymphocytes infiltrated into the epidermis with spongiosis. Immunohistochemically, IgE and IL-4 were present in IFN- $\gamma$ <sup>-/-</sup> mice presumably in association with mast cells and T cells, respectively (Figure 5b).

Epidermal cell suspensions, including epidermotropic lymphocytes as well as ECs, were prepared from the challenged skin and subjected to real-time PCR analysis for two chemokine receptors. CXCR3 is expressed mostly on Th1 cells, and CCR4 is preferentially on Th2 cells (Sebastiani *et al.*, 2002). Whereas CXCR3 was markedly expressed by FITC challenge in B6 but not in IFN- $\gamma$ <sup>-/-</sup> mice, CCR4 expression was dramatically increased by repeated FITC painting in IFN- $\gamma$ <sup>-/-</sup> but not in B6 mice (Figure 6). Taken together with the presence of IL-4 in IFN- $\gamma$ <sup>-/-</sup> mice challenged with FITC, this finding provides a possibility that IFN- $\gamma$  deficiency allows Th2 cells to infiltrate into the skin, leading to the occurrence of immediate and late-phase reactions.

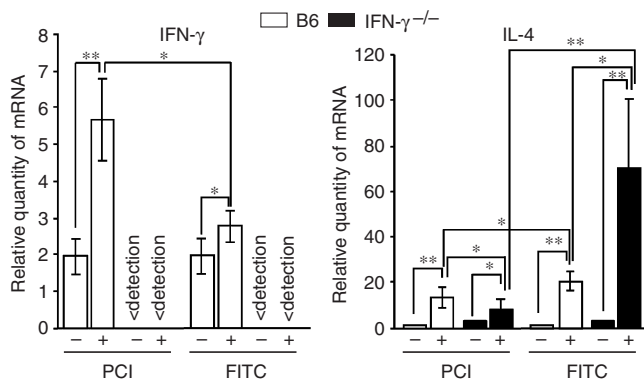


**Figure 4. Real-time PCR analysis of mRNA expression for cytokines in cervical lymph node cells.** B6 and IFN- $\gamma^{-/-}$  mice were sensitized with 5% PCI and challenged once with 0.5% PCI, or sensitized with 1% FITC and challenged six times with 0.5% FITC (twice a week for 3 weeks). Single-cell suspensions of immune cervical lymph node cells were prepared and subjected to real-time PCR analysis for IFN- $\gamma$  and IL-4. Data are expressed as the mean  $\pm$  SD of four mice. \* $P < 0.05$ , \*\* $P < 0.005$ .

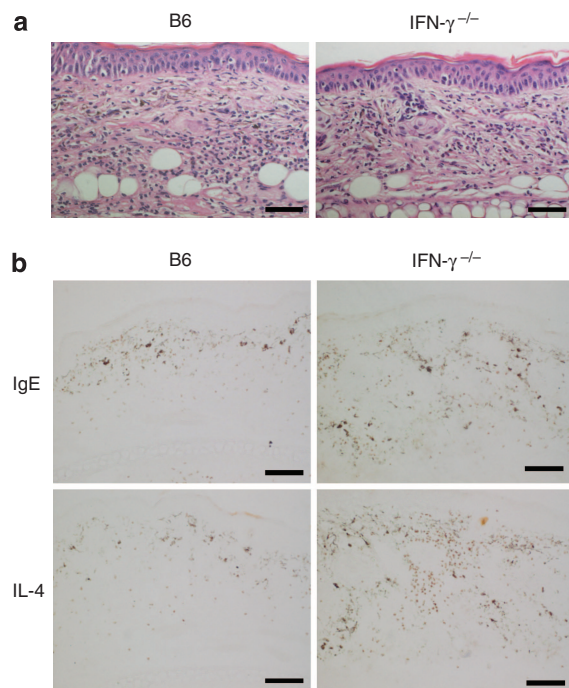


**Figure 5. Histology and immunohistochemical stainings for IgE and IL-4 in B6 and IFN- $\gamma^{-/-}$  mice receiving six times repeated elicitation with FITC.** (a) B6 and IFN- $\gamma^{-/-}$  mice were sensitized with 1% FITC and challenged with 0.5% FITC repeatedly six times (twice a week). The challenged ears were stained with hematoxylin and eosin. (b) Cryostat sections of the challenged ears were immunohistochemically stained with anti-IgE or anti-IL-4 mAb. Bar = 50  $\mu$ m.

#### Reduced CHS response to PCI in IFN- $\gamma^{-/-}$ mice receiving adoptive transfer

Draining lymph node cells ( $5 \times 10^6$  cells) from wild-type B6 mice that were sensitized with PCI 5 days before were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) and transferred to naive B6 or IFN- $\gamma^{-/-}$  mice.

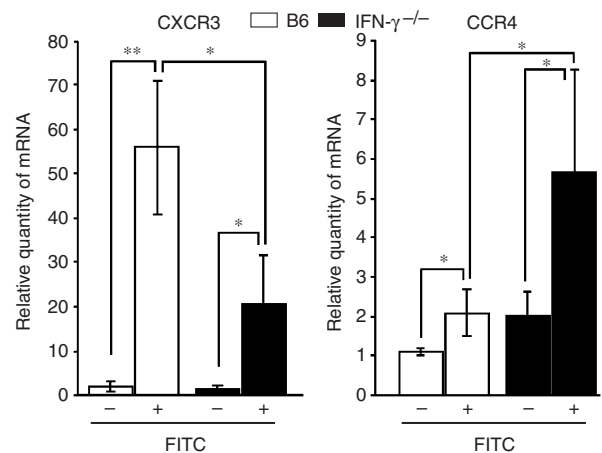


**Figure 6. Real-time PCR analysis of mRNA expression for chemokine receptors in skin-infiltrating cells.** B6 and IFN- $\gamma^{-/-}$  mice were sensitized with 5% PCI and challenged once with 0.5% PCI, or sensitized with 1% FITC and challenged six times with 0.5% FITC (twice a week for 3 weeks). Single-cell suspensions of epidermal and dermal cells were prepared and subjected to real-time PCR analysis for CXCR3 and CCR4. Data are expressed as the mean  $\pm$  SD of four mice. \* $P < 0.05$ , \*\* $P < 0.005$ .

Immediately after transfer, mice were challenged with PCI on their ears. Although B6 mice transferred with sensitized lymph node cells exhibited a significantly high ear swelling response, IFN- $\gamma^{-/-}$  mice failed to respond to the challenge (Figure 7a). This result clearly indicated impairment of the effluent phase of CHS in IFN- $\gamma^{-/-}$  mice. When the ears of mice receiving CFSE-labeled lymph node cells were histologically examined 24 hours after challenge, the number of CFSE-positive cells in the ears of IFN- $\gamma^{-/-}$  mice was significantly lower than that of B6 mice (Figure 7b).

To further confirm this paucity of infiltration, draining lymph node cells ( $5 \times 10^6$  cells) from green fluorescent protein (GFP) mice sensitized with DNFB 5 days before were transferred to naive B6 or IFN- $\gamma^{-/-}$  mice. The recipients were immediately challenged with DNFB. B6 but not IFN- $\gamma^{-/-}$  mice had a high ear swelling response (Figure 7c). Again, histological examination at 24 hours after challenge showed less infiltration of GFP-positive lymphocytes in IFN- $\gamma^{-/-}$  mice (Figure 7d).

To test the participation of IFN- $\gamma$  in the afferent limb, immune lymph node cells from B6 or IFN- $\gamma^{-/-}$  mice sensitized with PCI were transferred to naive B6 mice, and the recipient mice were challenged with PCI. The CHS response of recipients receiving sensitized lymph node cells of IFN- $\gamma^{-/-}$  mice was lower than that of mice receiving B6 lymph node cells (Figure 7e). This suggests that IFN- $\gamma$  is involved in both induction and elicitation of CHS.

#### Reduction of Th1 chemokine production by keratinocytes in IFN- $\gamma^{-/-}$ mice and enhancement of Th2 chemokine production by LCs in IFN- $\gamma^{-/-}$ mice

Epidermal cell suspensions were obtained from the ears of B6 and IFN- $\gamma^{-/-}$  mice that were challenged once with PCI or six times with FITC. Crude ECs were analyzed for EC-derived chemokines. Both challenges with PCI and FITC elevated the

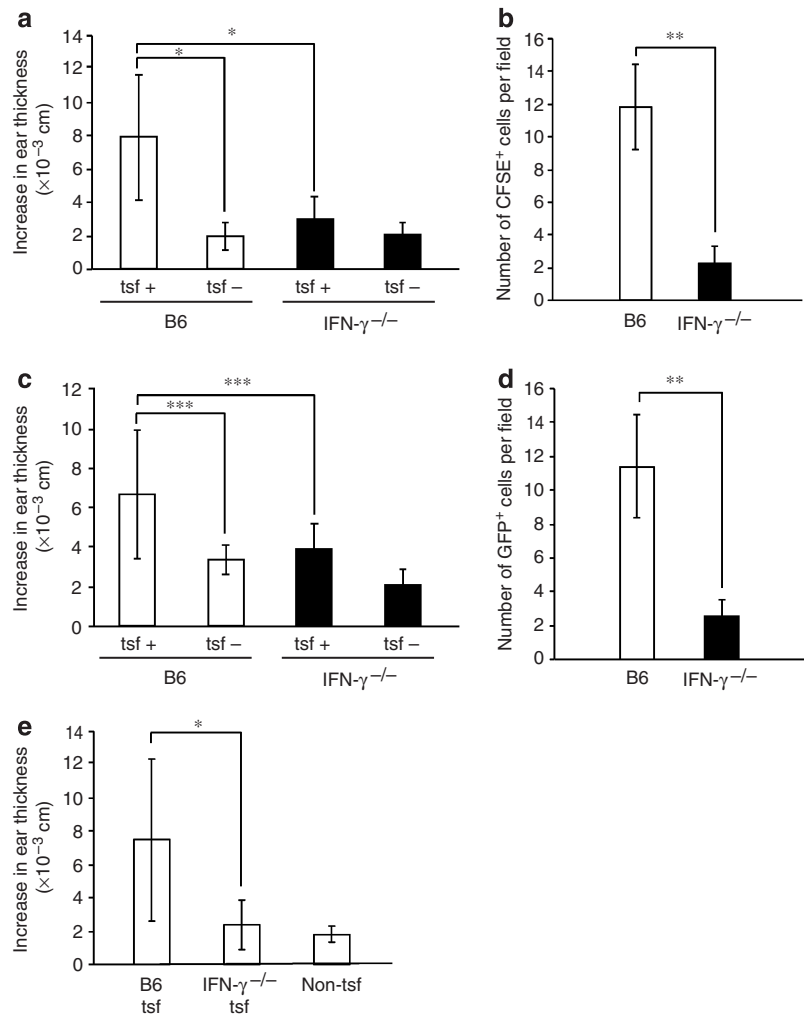


**Figure 7. Adoptive transfer of CHS using immune lymph node cells labeled with CFSE and those from GFP mice.** (a) Axillary, bradial, and inguinal lymph node cells ( $5 \times 10^6$  cells) of B6 mice that were sensitized 5% PCI on the shaved abdomen 5 days before were labeled with CFSE and transferred to naive B6 or IFN- $\gamma^{-/-}$  mice. Immediately after transfer (tsf), mice were challenged with 10  $\mu$ l of 0.5% PCI to both sides of ears (tsf +). Non-transferred B6 and IFN- $\gamma^{-/-}$  mice were described as tsf-. The increment of ear swelling was measured at 24 hours. (b) Ears of B6 and IFN- $\gamma^{-/-}$  mice receiving transfer of CFSE-labeled immune lymph node cells from PCI-sensitized mice were taken 24 hours after transfer and subjected to the histological study. The number of CFSE-positive cells in the dermis was counted in six different original magnification  $\times 200$  power fields and represented by the mean. (c) Immune lymph node cells were prepared from GFP mice sensitized with 0.5% DNFB and transferred to naive B6 or IFN- $\gamma^{-/-}$  mice. Mice were challenged with 10  $\mu$ l of 0.3% DNFB to both sides of ears (tsf +). Non-transferred mice were represented as tsf-. The increment of ear swelling was measured at 24 hours. (d) Ears of B6 and IFN- $\gamma^{-/-}$  mice receiving transfer of immune lymph node cells ( $5 \times 10^6$  cells) from DNFB-sensitized GFP mice were taken 24 hours after transfer and subjected to the histological study. The number of GFP-positive cells in the dermis was counted in six different original magnification  $\times 200$  power fields and represented by the mean. (e) Lymph node cells were prepared from B6 or IFN- $\gamma^{-/-}$  mice sensitized with 5% PCI and transferred to naive B6 mice. The recipient mice were challenged with 10  $\mu$ l of 0.5% PCI (B6, IFN- $\gamma^{-/-}$ ). The non-transferred mice represent the group of mice challenged alone. The ear thickness change was measured at 24 hours. Data are expressed as the mean  $\pm$  SD of four mice. \* $P < 0.001$ , \*\* $P < 1.0 \times 10^{-6}$ , and \*\*\* $P < 0.05$ .

expression of Th1 chemokine CXCL9 and CXCL10 in B6 mice, whereas such an augmentation was not found in IFN- $\gamma^{-/-}$  mice (Figure 8, left). As for Th2 chemokines, PCI challenge induced the expression of CCL17 and CCL22 in both B6 and IFN- $\gamma^{-/-}$  mice. Repeated FITC challenge augmented the expression of Th2 chemokines CCL17 and CCL22 in IFN- $\gamma^{-/-}$ , but FITC augmentation for CCL17 and CCL22 was minimal in B6 mice. It was reported that CCL27/CTACK-CCR10 signaling is an important regulator for skin immune inflammation (Homey *et al.*, 2002). Therefore, CCL27 expression was also monitored, but there was no

substantial difference between challenged and non-treated ears, or between B6 and IFN- $\gamma^{-/-}$  mice. Only marginal increment was observed in IFN- $\gamma^{-/-}$  mice that were challenged six times with FITC compared to non-treated IFN- $\gamma^{-/-}$  mice (relative quantitative mRNA: 3.1 vs 1). Accordingly, no enhanced expression of CCR10, a ligand for CCL27, was found in PCI- or FITC-challenged skin of B6 or IFN- $\gamma^{-/-}$  mice.

When CD11c<sup>+</sup> cells, namely LCs in the epidermis, were depleted from crude EC suspensions, and again subjected to real-time PCR analysis, signals of Th2 chemokines

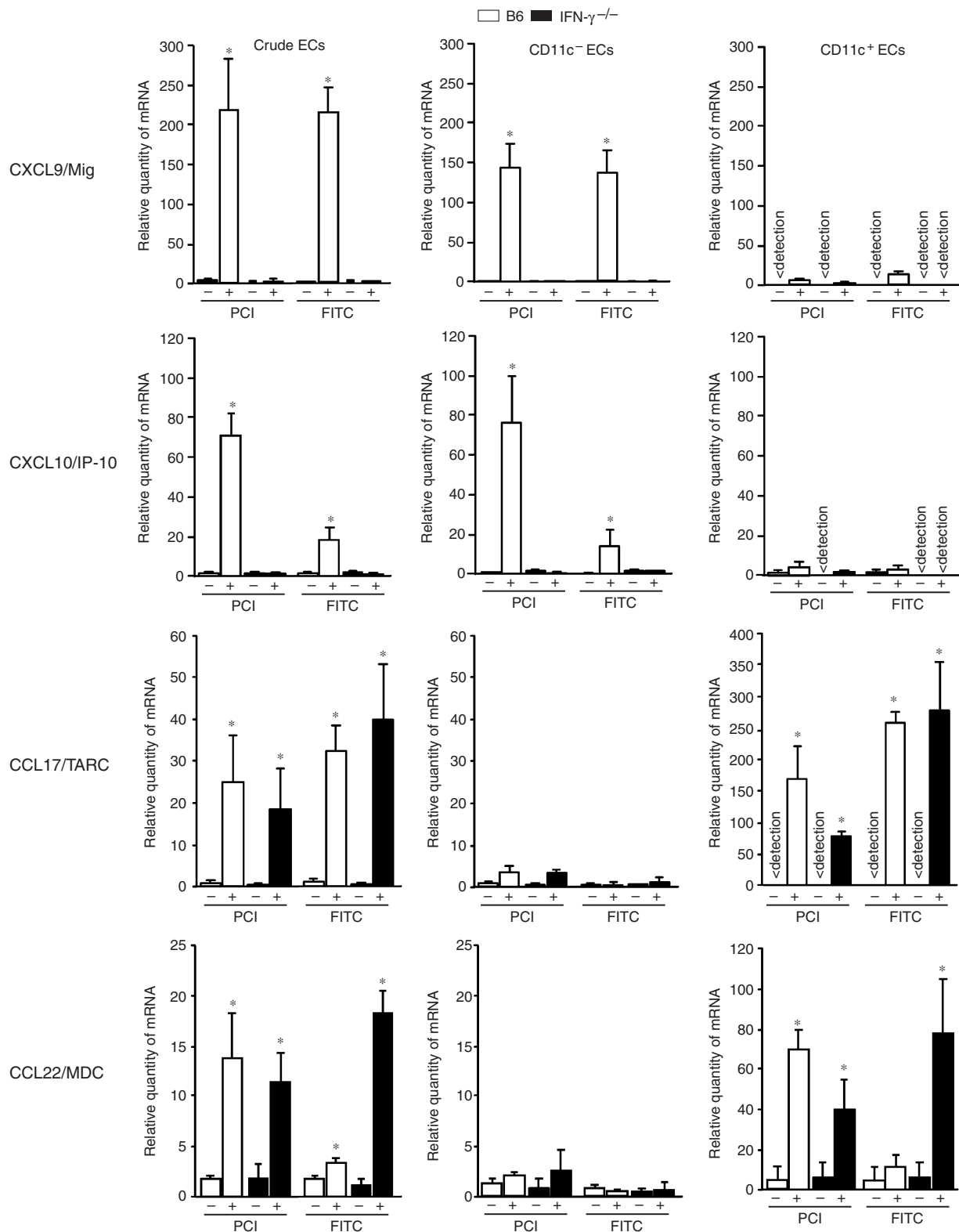


**Figure 8. Real-time PCR analysis of mRNA expression for chemokines in ECs.** B6 and IFN- $\gamma$ <sup>-/-</sup> mice were sensitized with PCI and challenged once with PCI, or sensitized with FITC and challenged six times with FITC (twice a week for 3 weeks). Single-cell suspensions of epidermal cells were prepared from the challenged ears. Unfractionated (crude ECs), CD11c<sup>+</sup> cell-depleted (CD11c<sup>-</sup>), and CD11c<sup>+</sup>-purified ECs were subjected to real-time PCR analysis for Th1 chemokines (CXCL9 and CXCL10) and Th2 chemokines (CCL17 and CCL22). The expression of mRNA is represented as fold increase ( $2^{-\Delta\Delta C_t}$ ), where  $\Delta\Delta C_t = [\Delta C_t(\text{sample})] - [\Delta C_t(\text{B6 CD11c}^- \text{ ECs without treatment})]$ , and  $\Delta C_t = [C_t(\text{sample})] - [C_t(\beta\text{-actin})]$ . Data are expressed as the mean  $\pm$  SD of four mice. \* $P < 0.03$ , compared between treated B6 mice or IFN- $\gamma$ <sup>-/-</sup> mice, and no treatment B6 mice.

disappeared, but those of Th1 chemokines remained (Figure 8, middle). Therefore, it was considered that Th1 chemokines are produced by keratinocytes, whereas Th2 chemokines are derived mainly from LCs.

The expression of Th2 chemokines, but not Th1 chemokines, in LCs was confirmed with CD11c<sup>+</sup> cell-purified ECs. LCs from FITC or even PCI-challenged mice expressed Th2 chemokines but not Th1 chemokines (Figure 8, right). The results indicated that IFN- $\gamma$  is required for keratinocyte expression of Th1 chemokines, but suppressive for LC expression of Th2 chemokines. IFN- $\gamma$ <sup>-/-</sup> mice had the decreased ability of keratinocytes to produce Th1 chemokines even by PCI challenge, but retained the ability of LCs to produce Th2 chemokines particularly upon repeated FITC challenge.

## DISCUSSION

In this study, we chose two distinct experimental systems to discriminate Th1 and Th2 cutaneous hypersensitivity reactions. The ordinary delayed-type CHS to PCI was used as a Th1-mediated response, and the immediate or late-phase reaction to repeatedly challenged FITC was used as a Th2-mediated response. We investigated the differences between the wild-type and IFN- $\gamma$ <sup>-/-</sup> mice in these two cutaneous hypersensitivity systems. IFN- $\gamma$ <sup>-/-</sup> mice were low responders in DTH to PCI, but high responders in the immediate and late-phase responses to FITC. The Th1/Th2 dichotomy-related immunological status was confirmed by the IFN- $\gamma$  expression in PCI-challenged wild-type mice and the IL-4 expression in FITC-challenged IFN- $\gamma$ <sup>-/-</sup> mice.

The depression of DTH to PCI and the enhancement of immediate and late-phase responses to FITC in IFN- $\gamma$ <sup>-/-</sup> mice are not particularly surprising, because the Th2-preponderant immunological balance in these mice is easily expected as a result of a relief from suppression of Th2 cells by IFN- $\gamma$ . In addition, participation of IFN- $\gamma$  in both afferent and efferent limbs duly results in depressed DTH. However, as inflammatory cells did not infiltrate in the PCI-challenged ears of IFN- $\gamma$ <sup>-/-</sup> mice, the observed downmodulation of cutaneous DTH could not be simply attributable to the systemic immunological shift to Th2 cells or to the impaired antigen-presenting function of cutaneous dendritic cells, but the failure in infiltration of T cells to the skin might be causative. Conversely, the extent of inflammatory cell infiltrate in FITC-challenged IFN- $\gamma$ <sup>-/-</sup> mice was high. Therefore, chemokines produced by ECs appeared to be critical for determining the magnitude of these cutaneous responses.

Among ECs, keratinocytes and LCs are the essential sources of chemokines (Sebastiani *et al.*, 2002). CXCR3<sup>+</sup> Th1 cells infiltrated in the PCI-challenged B6 mice, whereas CCR4<sup>+</sup> Th2 cells were seen in the FITC-challenged IFN- $\gamma$ <sup>-/-</sup> mice. Our real-time PCR analysis of EC-derived chemokines revealed that Th1 chemokines, CXCL9 and CXCL10, are produced by keratinocytes, and Th2 chemokines, CCL17 and CCL22, are mainly exposed by LCs. In IFN- $\gamma$ <sup>-/-</sup> mice, the Th1 chemokines were not expressed by PCI challenge, whereas the expression of Th2 chemokines retained when challenged with PCI or FITC. Therefore, it is likely that IFN- $\gamma$

is necessary for the production of Th1 chemokines by keratinocytes, but unnecessary or rather suppressive for the elaboration of Th2 chemokines by LCs. It is considered that the lack of development of cutaneous DTH to PCI in IFN- $\gamma$ <sup>-/-</sup> mice stems from the inability of keratinocytes to produce Th1 chemokines. In accordance with this finding, our transfer study using immune lymph node cells obtained from sensitized B6 mice and labeled with CFSE and those from sensitized GFP mice demonstrated low infiltration of transferred lymphocytes in IFN- $\gamma$ <sup>-/-</sup> mice.

The role of IFN- $\gamma$  for the production of CXCL9 and CXCL10 by keratinocytes has been reported (Albanesi *et al.*, 2000b; Mahalingam *et al.*, 2001; Sebastiani *et al.*, 2002). CCL17 and CCL22 have also been reported to be released from keratinocytes (Sebastiani *et al.*, 2002), and a combination of IFN- $\gamma$  and TNF- $\alpha$  is one of the most effective stimuli for keratinocytes to produce CCL17 and CCL22 (Kobayashi *et al.*, 2004). However, this study demonstrated that keratinocytes are not the main source of these Th2 chemokines, but that LCs are the producers. In accordance with this notion, cultured normal human keratinocytes cannot produce CCL17 even under stimulation with IFN- $\gamma$  and TNF- $\alpha$  (Tsuda *et al.*, 2003). It has been reported that LCs produce or express various chemokines, including CCL17, CCL22, CXCL11, and CCL5 (Fujita *et al.*, 2004, 2005). Cytokines such as TNF- $\alpha$ , GM-CSF, IL-1 $\beta$ , and IL-4 stimulate LCs to produce CCL17 and CCL22 (Soumelis *et al.*, 2002). It is notable that IFN- $\gamma$  is not a requirement for LCs to produce CCL17 and CCL22 but rather suppressive for their production (Xiao *et al.*, 2003). In IFN- $\gamma$ <sup>-/-</sup> mice, therefore, Th2 cells are prone to infiltrate in the dermis, and the late-phase reaction can occur as a result of successful migration of Th2 cells to the skin. In this context, it is an interesting issue that the infiltrating lymphocytes showed epidermotropism possibly because of Th2 chemokine production by LCs. The production of the chemokines by LCs may support the recent concept that LCs are rather regulatory for the development of CHS (Kaplan *et al.*, 2005; Kissenpfennig *et al.*, 2005).

Interferon- $\gamma$ , as well as IL-12 (Wolf *et al.*, 1994), is the key cytokine for Th1 responses. Furthermore, recent accumulating evidence has suggested the important role of IL-17 for CHS (Nakae *et al.*, 2002; He *et al.*, 2006). As IL-17 stimulates keratinocytes to release certain cytokines and chemokines with synergistic or additive effects when used together in IFN- $\gamma$  (Albanesi *et al.*, 2000a), our results in IFN- $\gamma$ <sup>-/-</sup> mice possibly stem from the relief of the synergistic effects. Our study suggests that IFN- $\gamma$  has a mandatory role for conditioning the skin milieu by modulating Th1 and Th2 chemokines.

## MATERIALS AND METHODS

### Animals and chemicals

Eight to 10-week-old female B6 mice were purchased from Japan SLC (Hamamatsu, Japan) and Kyudo Co. (Tosu, Japan). B6 and IFN- $\gamma$ <sup>-/-</sup> mice were obtained from Jackson Laboratory (Bar Harbor, ME), and GFP mice were obtained from Japan SLC. These mice were maintained in the Laboratory Animal Research Center in University of Occupational and Environmental Health under specific pathogen-free conditions. Female mice were used in this study. All animal experiments were performed according to the guidelines for the care

and use of animals approved by our university. PCI was purchased from Tokyo Kasei (Tokyo, Japan), FITC from Sigma Chemical Co. (St Louis, MO), and DNFB from Nakalai Tesque (Kyoto, Japan).

### Sensitization and elicitation of CHS

For induction of CHS to PCI, mice were painted on the clipped abdomen with 50  $\mu$ l of 5% PCI in ethanol/acetone (3:1). Five days after sensitization, mice were challenged by painting both sides of each ear with 20  $\mu$ l of 0.5% PCI in ethanol/acetone (3:1). The ear thickness of mice was measured using dial thickness gauge (Ozaki Co., Tokyo, Japan) before and 24 hours after challenge. Ear swelling was calculated as (ear thickness after challenge)–(ear thickness before challenge).

For induction of CHS to FITC, mice were painted on the clipped abdomen with 200  $\mu$ l of 1% FITC in acetone/di-*n*-butylphthalate (1:1). Five days after sensitization, mice were challenged by painting both sides of each ear with 20  $\mu$ l of 0.5% FITC in acetone/di-*n*-butylphthalate (1:1) twice a week for 3 weeks. The ear thickness of mice was measured using dial thickness gauge before, 0, 1, 4, and 24 hours after challenge.

For induction of CHS to DNFB, mice were painted on the clipped abdomen with 50  $\mu$ l of 0.5% DNFB in acetone/olive oil (4:1). The ears were challenged with 20  $\mu$ l of 0.3% DNFB in acetone/olive oil (4:1).

### Preparation of ECs, CD11c<sup>+</sup> cell-depleted ECs, purified CD11c<sup>+</sup> ECs, and lymph node cells

Ears were obtained from mice 24 hours after the last challenge with PCI or FITC, and epidermal and dermal skin was obtained by removing cartilage and subcutaneous tissues. After incubation for 1 hour at 37 °C in a 0.2% solution of trypsin in PBS, the epidermis was separated from the dermis, and ECs were dispersed in PBS supplemented with 10% fetal calf serum (Gibco, Carlsbad, CA) by rubbing the separated epidermal sheets (Tokura *et al.*, 1994). The cells were filtered through a cell strainer and washed twice in PBS. In some experiments, CD11c<sup>+</sup> ECs were purified with automagnetic-activated cell sorting (Miltenyi Biotec, Bergisch Gladbach, Germany). After ECs were incubated with RPMI 1640 supplemented with 10% fetal calf serum for 30 minutes, ECs were first reacted with CD11c microbeads (120-000-322; Miltenyi Biotec) for 30 minutes and selected for CD11c<sup>+</sup> ECs, representing LCs. The purity of CD11c<sup>+</sup> cells was 70–80% (Sugita *et al.*, 2007). After removing CD11c<sup>+</sup> cells, CD11c<sup>–</sup> ECs were obtained and CD11c<sup>+</sup> cells were less than 0.1%. Among CD11c-positive fractionated cells from ECs, more than 99% of CD11c<sup>+</sup> cells were Langerin (eBioscience, San Diego, CA) positive (data not shown).

B6 and IFN- $\gamma$ <sup>–/–</sup> mice were sensitized with 5% PCI and challenged once with 0.5% PCI, or sensitized with 1% FITC and challenged six times with 0.5% FITC (twice a week for 3 weeks). Single-cell suspensions of immune cervical lymph node cells were prepared from the treated mice.

### Histological assessment

Twenty-four hours after challenge with PCI and FITC, mice were killed by cervical dislocation and ears were amputated. The ears were fixed with formalin and embedded in paraffin. Two- to three-micrometer-thick sections were cut and stained with hematoxylin and eosin. An indirect immunohistochemical staining was

performed. The first antibodies included anti-CD3 (clone KT3, rat IgG2a; Chemicon, Billerica, MA), anti-IgE (clone 23G3, rat IgG1 $\kappa$ ; eBioscience), and IFN- $\gamma$  (clone XMG1.2, rat IgG1 $\kappa$ ; eBioscience) and mAbs. A goat anti-rat IgG antibody conjugated with horseradish peroxidase was used as the second antibody, and reaction was visualized with diaminobenzidine.

### Adoptive transfer

B6 mice were sensitized with 50  $\mu$ l of 5% PCI in ethanol/acetone (3:1) on the clipped abdomen. Five days after sensitization, the axillary, brachial, and inguinal lymph nodes were removed, single-cell suspensions were prepared and labeled with 6.5  $\mu$ M CFSE (Molecular Probes, Eugene, OR). The cells resuspended in RPMI containing 0.2% fetal calf serum were injected intravenously to naive B6 mice or naive IFN- $\gamma$ <sup>–/–</sup> mice ( $5 \times 10^6$  cells per mouse). Immediately after the cell transfer, the ears were challenged with 20  $\mu$ l of 0.5% PCI in ethanol/acetone (3:1), and the increase in ear swelling was measured 24 hours later. In parallel, ears of transferred B6 and IFN- $\gamma$ <sup>–/–</sup> mice were histologically examined 24 hours after cell transfer. CFSE-positive cells in about 7  $\mu$ m ear section of each mouse were counted.

In another transfer study, GFP mice were sensitized with 50  $\mu$ l of 0.5% DNFB in acetone/olive oil (4:1). Five days later, the immune lymph node cells were prepared as above and injected intravenously to naive B6 mice or naive IFN- $\gamma$ <sup>–/–</sup> mice ( $5 \times 10^6$  cells per mouse). The ears were challenged with 20  $\mu$ l of 0.3% DNFB in acetone/olive oil (4:1). The ear swelling response, histology, and GFP-positive cells were examined as above.

### Real-time quantitative PCR

Total cellular RNA was extracted with an RNA extraction kit (Promega, Madison, WI) from freshly prepared skin samples. RNA was then reverse-transcribed and amplified by random hexamer in single-tube assay using the TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA) with gene-specific sense and antisense primers and a detection probe labeled on the 5'-end with the reporter dye 6-FAM. Primers and probes were obtained from TaqMan Gene Expression Assays Inventories (Applied Biosystems). Using an ABI Prism 7000 Sequence Detection Systems (Applied Biosystems), duplicate samples were reverse-transcribed and amplified under the following consecutive steps: 2 minutes at 50 °C, 10 minutes at 95 °C, followed by 50 amplification cycles of 15 seconds at 95 °C and 1 minute at 60 °C. Sequence-specific amplification was detected as an increased fluorescent signal of 6-FAM exceeding the threshold limit during the amplification cycle. Quantification of gene-specific message levels was determined by comparing fluorescence intensity from unknown RNA samples to the fluorescence intensity of standard curve generated from control mRNA levels. Amplification of the gene for mouse  $\beta$ -actin was performed on all samples to control interspecimen variations in RNA amounts. The result for each gene was normalized to the quantity of mouse  $\beta$ -actin detected in the sample. Levels of gene-specific message were graphed as normalized message units as determined from standard curve.

### Statistical analysis

Data were analyzed using an unpaired two-tailed *t*-test. *P* < 0.05 was considered to be significant.

## CONFLICT OF INTEREST

The authors state no conflict of interest.

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