



Mouse Model of Hydroquinone Hypersensitivity via Innate and Acquired Immunity and its Promotion by Combined Reagents

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We established a mouse model of contact hypersensitivity (CHS) to hydroquinone (HQ), a widespread chemical in our environment. HQ was painted onto flanks; then, HQ was challenged by painting onto ear pinnae on days 7 and 14. The CHS after the second challenge was markedly greater than that after the first challenge. Both challenges increased thymic stromal lymphopoietin and T helper type 2 cytokines in ear pinnae, whereas IFN- γ (typical T helper type 1 cytokine) was decreased, despite an increase in IL-18 (typical IFN- γ inducer). In nude mice (T cell-reduced), although a first challenge induced CHS, a second challenge did not augment it. In severe combined immunodeficient, severe combined immunodeficient-beige, and IL-1–deficient mice, CHS was not induced. However, CHS was inducible in severe combined immunodeficient-beige mice after transfer of natural killer cells from HQ-sensitized normal mice. Tretinoin (used for enhancing the skin-whitening effect of HQ) and resin monomers (used to prevent polymerization of HQ) lowered the HQ concentration needed to establish sensitization to HQ. The augmented CHS after a second challenge was reduced by JNJ7777120, dexamethasone, supatast tosilate (T helper type 2-cytokine inhibitor), and anti-thymic stromal lymphopoietin antibody. These results suggest that (i) thymic stromal lymphopoietin, IL-1, and T and/or natural killer cells are important in establishing and augmenting CHS to HQ and (ii) inflammatory chemicals may promote CHS to HQ as adjuvants.

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INTRODUCTION

We are frequently exposed to hydroquinone (HQ) through diet, skin contact, and inhalation (Astaneh et al., 2005; Deisinger et al., 1996; Stanfield et al., 2006). HQ is a nonvolatile chemical used in the photographic, rubber, and chemical industries, and it is also present in cigarettes and their smoke, as well as in diesel-engine exhaust. HQ is found in natural agents, too, because it is a metabolite of benzene. Moreover, HQ is frequently added to chemical liquids or emulsions as a stabilizer (e.g., to dental resin monomers to prevent their polymerization). Since the 1950s, high concentrations of HQ

(1–10%) have been used as skin-lightening agents for hyperpigmentation (including melasma, solar lentigines, and dyschromia). HQ has also been used in combination with tretinoin (vitamin A derivative) because this combination enhances the whitening effect (Astaneh et al., 2005).

However, it should be noted that HQ has various detrimental effects, such as irritant dermatitis, skin redness, exogenous ochronosis, and hypopigmentation, on its users, including photographic developers (Nordlund et al., 2006). Thus, the over-the-counter sale of HQ in high concentrations (over 2%) has recently been banned.

HQ is not a common cause of allergic contact hypersensitivity (CHS), despite its widespread use and the high potential for exposure (Nordlund et al., 2006). Indeed, Belina and Graupe (1991) reported only one case among 65 patients treated with HQ, and Grimes (2004) reported possible allergic contact reactions in two of 25 patients. However, it seems likely that a patch test is not sufficient to confirm CHS to HQ. Because irritant reactions are the most common, it is also likely that patients with HQ-induced irritation include some patients with CHS to HQ. In any event, the incidence of allergic CHS to HQ is currently not clear. Ewens et al. (1999) reported in mice that after subcutaneous injection into hindfoot pads, HQ can act as a hapten to increase cell numbers in popliteal lymph nodes. Basketter et al. (2007) reported that HQ is classified as a strong sensitizer by a local lymph node assay (LLNA), which measures the concentration of a chemical to produce a 3-fold stimulation of

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Abbreviations: CHS, contact hypersensitivity; EC3, estimated concentration needed to produce a stimulation index of 3; HEMA, 2-hydroxyethyl methacrylate; HQ, hydroquinone; KO, knockout; LLNA, local lymph node assay; MMA, methyl methacrylate; NK, natural killer; OX, oxazolone; SCID, severe combined immunodeficient; Th, T helper; TNF, tumor necrosis factor; TSLP, thymic stromal lymphopoietin

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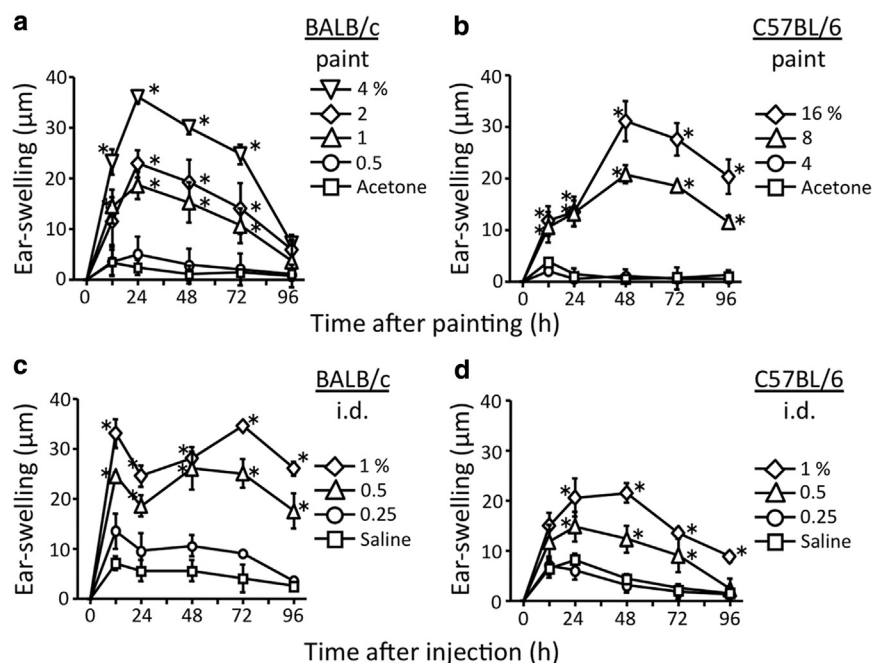


Figure 1. Direct inflammatory effects of HQ itself in BALB/c and C57BL/6 mice. (a, b) HQ in acetone was painted at the indicated concentrations onto ear pinnas (10 μ l/each side of the ear). (c, d) HQ was dissolved in saline and intradermally injected into ear pinnas at the indicated concentrations (20 μ l/ear). Each value is the mean $\pm$ standard deviation from 6 ears (i.e., 3 mice). * $P < 0.01$ versus acetone (a and b) or versus saline (c and d) at the same time point. The results shown for a given experiment were confirmed by repeating the experiment at least once. h, hours; HQ, hydroquinone; i.d., intradermal.

murine lymph node cell proliferation. However, there have been few experimental reports of HQ-induced allergies, and no one has confirmed that HQ can indeed induce allergic CHS. On the contrary, in vitro experiments suggest that HQ may be immunosuppressive because (i) HQ impairs the proliferation of lymphocytes and causes DNA damage in mononuclear cells in vitro (Fabiani et al., 2001; Li et al., 1996; McCue et al., 2003) and (ii) HQ suppresses the production of IL-1 β , IFN- γ , and tumor necrosis factor (TNF)- α in human peripheral blood monocytes (Ouyang et al., 2000). Thus, HQ-induced CHS has not been sufficiently well characterized. In this study, we established a mouse model of the CHS induced by HQ and clarified its characteristics. We also examined the effects of combinations of HQ with tretinoin and resin monomers.

RESULTS

Direct inflammatory effects of HQ in BALB/c and C57BL/6 mice

We first examined whether HQ has inflammatory effects in BALB/c and C57BL/6 mice. Painting HQ of 1% or greater concentration onto ear pinnas induced concentration-dependent ear swelling in BALB/c (Figure 1a), but even 4% HQ was not inflammatory in C57BL/6 (although concentrations of 8% and 16% were) (Figure 1b). Intradermal injection of 0.5% HQ into ear pinnas also induced ear swelling at the injection site in both BALB/c and C57BL/6 mice (Figure 1c and d). However, the difference between BALB/c and C57BL/6 was smaller than that seen after painting. These results indicate that BALB/c mice are more sensitive to HQ than C57BL/6 mice.

HQ induces CHS

We first examined the skin-sensitizing potential of HQ by a LLNA (see Materials and Methods). The concentration of a chemical to produce a 3-fold stimulation of murine lymph node

cell proliferation (estimated concentration needed to produce a stimulation index of 3 [EC3]) was determined as described by Basketter et al. (2000). EC3 of HQ was 0.29% in an experiment using BALB/c mice (Figure 2a). However, the response of C57BL/6 mice to HQ was too low to determine its EC3 under the condition used in this experiment (Figure 2a). In BALB/c mice, the EC3 of HQ was smaller than that of isoeugenol and larger than that of oxazolone (OX) (Figure 2b), suggesting that the sensitizing potency is OX > HQ > isoeugenol. These results are consistent with those reported by Gerberick et al. (2004) and Basketter et al. (2007), in which LLNA was carried out using CBA mice.

Next, we examined whether HQ does indeed induce hypersensitivity in mice. Mice were treated with HQ by painting different concentrations onto shaved flanks (100 μ l/mouse) (sensitization step), and 7 days later their ear pinnas were challenged with one of several concentrations of HQ (10 μ l/each side of the ear) (elicitation step). Painting 4% HQ onto flanks was not particularly inflammatory. When BALB/c mice were challenged with 0.5% HQ, ear swelling was induced in mice treated at the sensitization step with 0.25% or more HQ but not with 0.125% HQ (Figure 2c). On the other hand, ear swelling was induced in C57BL/6 mice only when they were treated with 16% HQ at the sensitization step and challenged with 4% HQ (Figure 2d). As shown in Figure 2e, when BALB/c mice were sensitized using 4% HQ, allergic ear swelling was induced by 0.5% HQ or more (but not by 0.25% HQ). In the following experiments, we used BALB/c mice.

Effects of repeated challenges on allergic ear swelling

Next, we examined the effects of repeated HQ challenges. Mice were sensitized to HQ by treating flanks with 4% HQ, and their ear pinnas were given a first and a second challenge with 0.5% HQ on days 7 and 14, respectively. The second challenge induced greater and more prolonged allergic ear swelling than the first (Figure 2f). An additional (i.e., third, on day 28) challenge induced allergic ear swelling even at

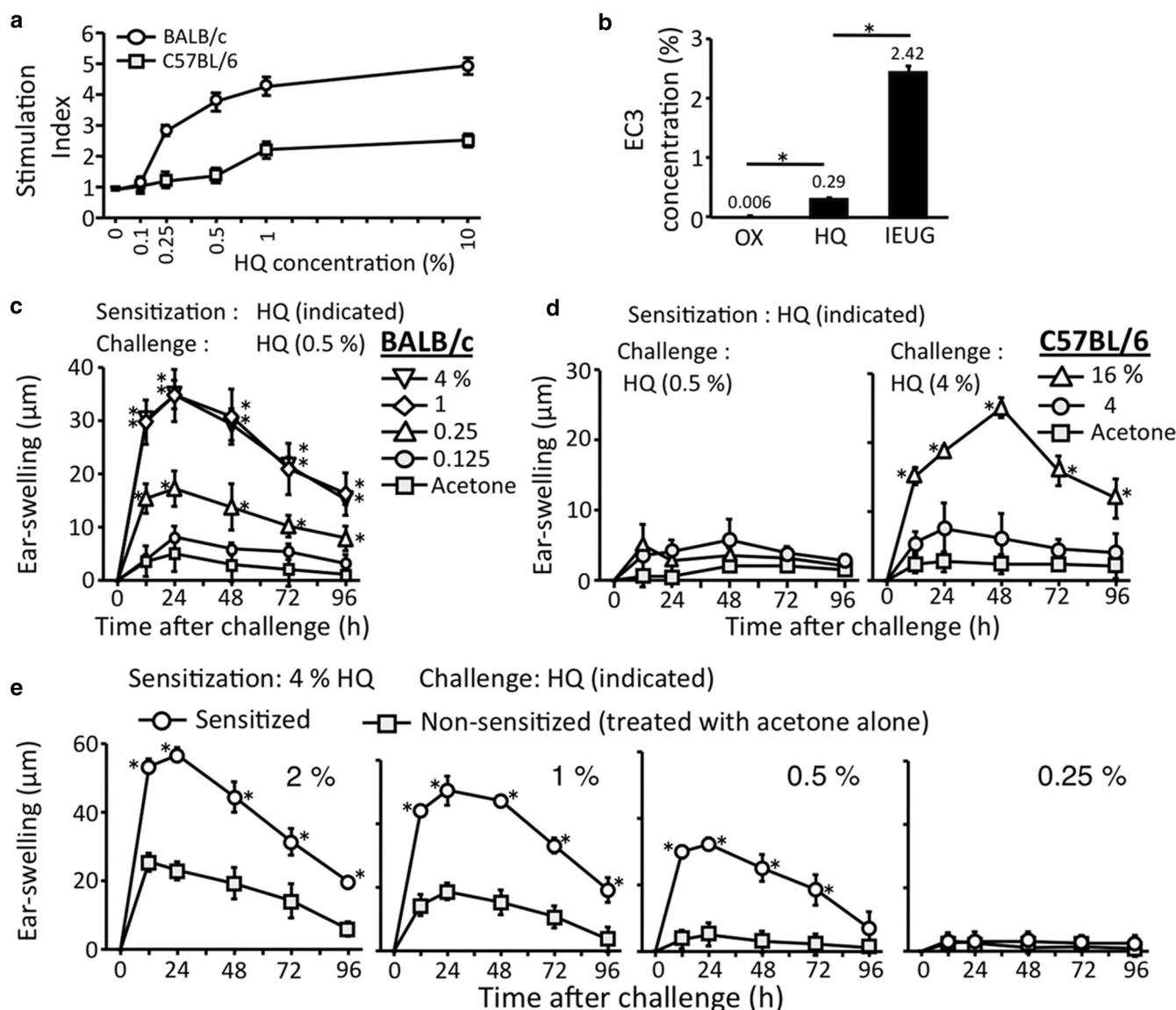


Figure 2. HQ induces allergic ear swelling. (a) Sensitizing potency of HQ. Indicated concentrations of HQ were painted onto ear pinnas daily for 3 consecutive days. A single intraperitoneal injection of BrdU was given on day 5. The auricular lymph nodes were removed on day 5. (b) Comparison of HQ sensitizing potency with those of well-known haptens by EC3 value in BALB/c mice. In this experiment, 0.1%, 0.25%, and 0.5% HQ; 1%, 2.5%, and 5% isoeugenol (IEUG); or 0.001%, 0.01%, and 0.1% OX were painted onto ear pinnas daily for three consecutive days. A single intraperitoneal injection of BrdU was given on day 4. The auricular lymph nodes were removed on day 5. * $P < 0.01$. (c, d) Effects of various concentrations of HQ on the sensitization step in BALB/c and C57BL/6 mice. The indicated concentrations of HQ were painted onto shaved flanks, and 7 days later ear pinnas were challenged by painting with 0.5% HQ. * $P < 0.01$ versus nonsensitized naïve mice. (e) Effects of various concentrations of HQ on the elicitation step in BALB/c mice. Shaved flanks were painted with 4% HQ, and 7 days later ear pinnas were challenged by painting with the indicated concentrations of HQ. (f) Effects of repeated challenges with HQ. Shaved flanks were painted with 4% HQ, and 7 days later ear pinnas were challenged with HQ (first challenge). Fourteen days later, a second challenge with HQ was given to the same mice, and 28 days later, these mice were given a third HQ challenge, this time at one of the indicated concentrations. The first and second challenges were carried out by painting 0.5% HQ onto ear pinnas. Each value is the mean $\pm$ standard deviation from six ears (i.e., three mice). * $P < 0.01$ versus nonsensitized naïve mice (or “acetone” in far right panel) at the same time point. (g) Effects of the timing of HQ challenge. BALB/c mice were painted with 4% HQ on shaved flanks, and 3, 5, 7, or 30 days later their ear pinnas were challenged with 0.5% HQ. Twenty-four hours after the challenge, ear swelling was measured. * $P < 0.01$ versus day 0. (h) Hapten-specific allergic ear swelling. Mice were painted with 4% HQ or 0.5% OX on shaved flanks. Seven days later, their ear pinnas were challenged (first challenge) by painting with 0.5% HQ or 0.5% OX (10 µl/each side of the ear), and 14 days later, they were again challenged (second challenge) by painting with 0.5% HQ or 0.5% OX (10 µl/each side of the ear). Ear swelling was measured 24 hours after the second challenge. * $P < 0.01$. (i) Effects of spleen cell transfer. Mice were painted with 4% HQ on shaved flanks. Seven days later, their ear pinnas were challenged by painting with 0.5% HQ. Five days after this challenge, spleens were taken, and 1×10^7 spleen cells were intravenously transferred into naïve (i.e., normal) mice. As a control, spleen cells from naïve mice were transferred into other naïve mice. Twenty-four hours after the cell transfer, the ear pinnas of the recipients were challenged by painting with 0.5% HQ, and ear swelling was measured 24 hours after the challenge. * $P < 0.01$. (j) Structures of the HQ isomers pyrocatechol (PC) and resorcinol (RR), and the oxidized HQ 1,4-benzoquinone (BQ). (k) Cross-reactivity among HQ, HQ isomers, and oxidized HQ. In this experiment, to obtain clear results, cross-reactivity was examined in mice whose sensitivity to HQ was enhanced by an HQ challenge. To this end, mice were painted with 4% HQ on their shaved flanks, and 7 days later their ear pinnas were challenged by painting with 0.5% HQ. Seven days after that challenge, their ear pinnas were again challenged by painting, this time with 0.5% HQ, 0.5% BQ, 0.5% PC, or 0.5% RR, and ear swelling was measured 24 hours after the second challenge. Mice without sensitization treatment served as a control. * $P < 0.01$. Throughout Figure 2, each value is the mean $\pm$ standard deviation from six ears (i.e., three mice). The results shown for a given experiment were confirmed by repeating the experiment at least once. BQ, 1,4-benzoquinone; EC3, estimated concentration needed to produce a stimulation index of 3; h, hours; HQ, hydroquinone; NS, not significant; OX, oxazolone; IEUG, isoeugenol; PC, pyrocatechol; RR, resorcinol.

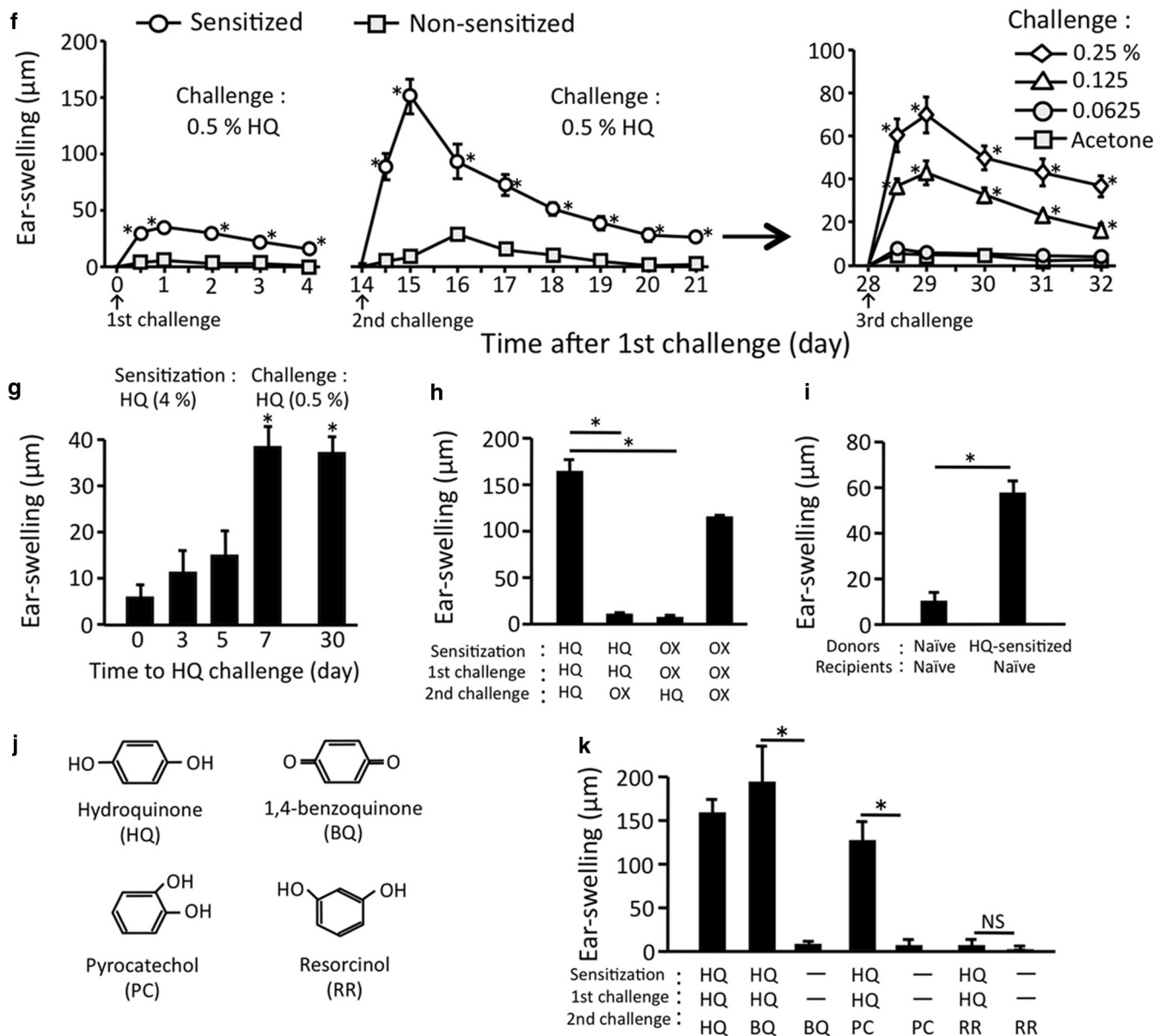


Figure 2. Continued

0.125% and 0.25% HQ (Figure 2f, right), although the first challenge did not induce such a marked ear swelling at these low concentrations (Figure 2c and e). Such augmentation did not occur in C57BL/6 (see Supplementary Figure S1 online). These findings indicate that repeating HQ challenges not only augments the magnitude of allergic ear swelling but also enhances the sensitivity to HQ in BALB/c mice.

HQ-induced CHS is hapten specific

As shown in Figure 2g, allergic ear swelling occurred (or tended to occur) at 5 to 7 days after the initial HQ exposure (i.e., painting onto flanks). HQ-induced allergic ear swelling occurred only in those mice previously exposed to HQ, not to OX (Figure 2h). In the cell-transfer experiment shown in Figure 2i, HQ-induced allergic ear swelling occurred in the mice into which spleen cells were transferred from mice sensitized to HQ but not in mice into which spleen cells were

transferred from naïve (normal) mice. These results clearly indicate that the HQ-induced allergic ear swelling is hapten specific.

Cross-reactivity among HQ isomers

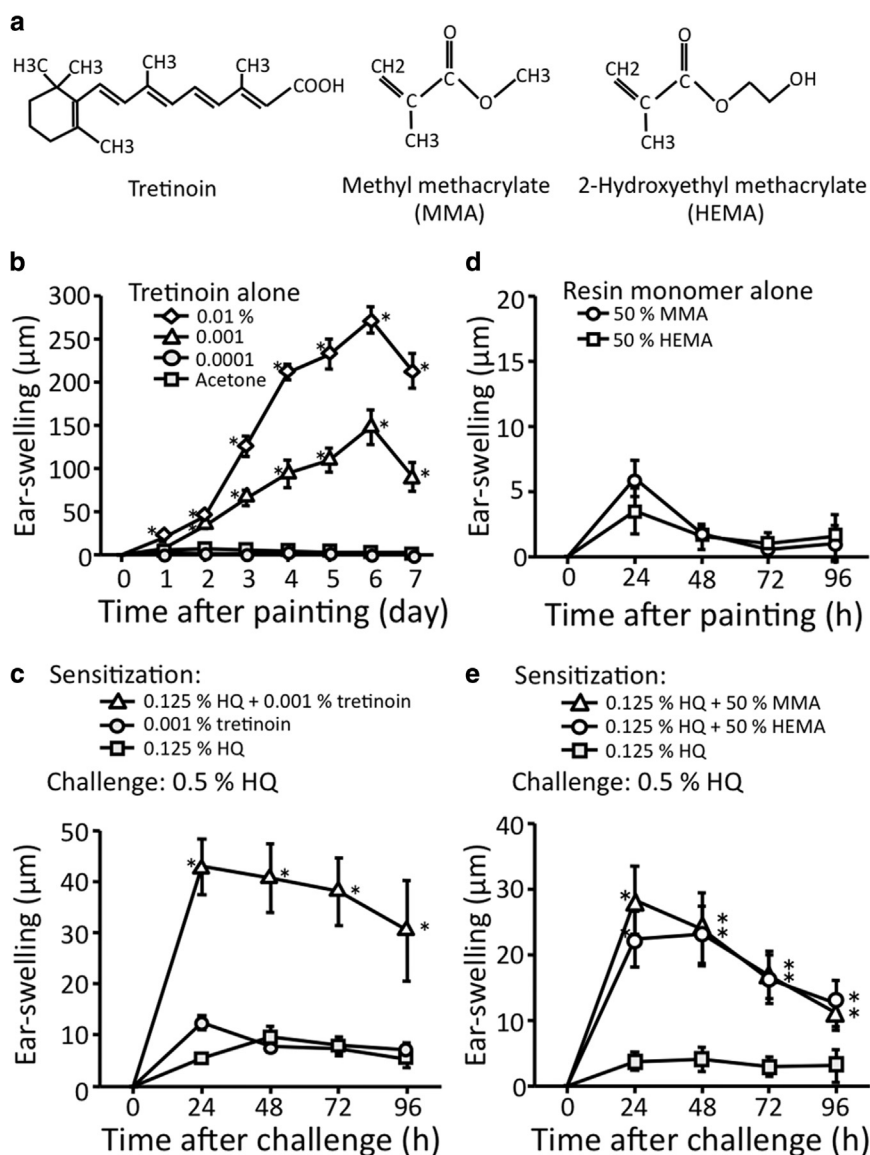
HQ has two isomers, pyrocatechol and resorcinol (Figure 2j). HQ is easily oxidized to yield 1,4-benzoquinone. These are used as medicines and chemicals, for example, as acne treatment, as hair colorants, and in rubber manufacture. In HQ-sensitized mice, 1,4-benzoquinone and pyrocatechol, but not resorcinol, induced allergic ear swelling (Figure 2k).

Augmentation of HQ-induced hypersensitivity by other chemicals

Inflammatory substances reportedly exert adjuvant effects in the establishment of the CHS induced by haptens (Grabbe et al., 1996), and HQ is often used in combination with other chemicals. For example, a combination of HQ with

Figure 3. Augmentation of HQ-induced hypersensitivity by other chemicals (as shown by the response to a first challenge). (a) Structures of tretinoin and resin monomers. (b) Direct inflammatory effects of tretinoin itself in BALB/c mice.

Tretinoin was dissolved in acetone, and the indicated concentrations were painted onto ear pinnas (10 μ l/each side of the ear). * P < 0.01 versus acetone at the same time point. (c) Effects of tretinoin at the sensitization step. The indicated mixture was painted onto shaved flanks (100 μ l/mouse), and 7 days later their ear pinnas were challenged by painting with 0.5% HQ (10 μ l/each side of the ear). * P < 0.01 versus 0.125% HQ and 0.001% tretinoin separately at the same time point. (d) Direct inflammatory effects of MMA and HEMA. A mixture of acetone and MMA or HEMA (1:1, i.e., 50% concentration) was painted onto ear pinnas (10 μ l/each side of the ear). (e) The indicated mixture was painted onto shaved flanks (100 μ l/mouse), and 7 days later their ear pinnas were challenged by painting with 0.5% HQ (10 μ l/each side of the ear). * P < 0.01 versus 0.125% HQ at the same time point. In panels b–e, each value is the mean $\pm$ standard deviation from six ears (i.e., three mice), and the results shown for a given experiment were confirmed by repeating the experiment at least once. h, hours; HEMA, 2-hydroxyethyl methacrylate; HQ, hydroquinone; MMA, methyl methacrylate.



tretinoin is used in cosmetics for reducing wrinkles and to enhance the skin-whitening effect of HQ. In addition, HQ is used as a stabilizer in dental resin monomers to prevent the polymerization of the resin monomers. We previously reported that resin monomers act as adjuvants to promote Ni-allergies (Bando et al., 2014). So, we examined whether tretinoin and resin monomers (Figure 3a) might have adjuvant effects on HQ-induced hypersensitivity. In these experiments, tretinoin and resin monomers, as well as HQ, were dissolved in acetone.

Effects of tretinoin on HQ-induced allergic ear swelling

As shown in Figure 3b, tretinoin itself, at concentrations of 0.001% or more, induced ear swelling after painting onto ears. In the next experiment, 0.001% tretinoin alone, 0.125% HQ alone, or a mixture of tretinoin (0.001%) and HQ (0.125%) (100 μ l/mouse) was painted onto shaved flanks, and 7 days later, ear pinnas were challenged by painting with 0.5% HQ (10 μ l/each side of the ear). As shown in Figure 3c, 0.125% HQ by itself did not establish sensitization to HQ.

However, marked allergic ear swelling was induced in mice sensitized with a mixture of tretinoin and 0.125% HQ. These results indicate that tretinoin has a potent adjuvant activity at the step at which sensitization occurs to HQ.

Effects of resin monomers

As shown in Figure 3d, methyl methacrylate (MMA) and 2-hydroxyethyl methacrylate (HEMA), when painted onto ear pinnas, produced only a transient mild swelling, even at 50% concentration. However, in mice in which flanks had been previously treated with a mixture containing 0.125% HQ and 50% MMA or HEMA, a challenge to their ear pinnas with 0.5% HQ did induce allergic ear swelling (Figure 3e). These results indicate that MMA and HEMA also exhibit an adjuvant activity at the step of sensitization to HQ.

Infiltration of cells into the allergic ear-swelling site

The ear pinnas of HQ-sensitized mice were given two challenges with 0.5% HQ (the first on day 7 and the second on day 14). Twenty-four hours after each challenge, redness and

vasodilation were observed (see [Supplementary Figure S2](#) online). The second challenge produced skin edema, vasodilation, and cell infiltrations (including mast cells) that were more severe than those induced by the first challenge ([Figure 4a–e](#)). Next, we examined the numbers of cells infiltrating the swelling sites. The second challenge induced greater accumulations of T cells, B cells, natural killer (NK) cells, macrophages, neutrophils, eosinophils, and basophils than the first challenge ([Figure 4f](#)). Serum total IgE and HQ-specific IgE, parameters that increase in T helper (Th) type 2 immune responses, increased with repeated challenges ([Figure 4h](#)).

Cytokine profiles in allergic sites after the first and second challenges

Next, we measured cytokines and histamine in the ear pinnae exhibiting allergic inflammation at 24 hours after the first and the second challenges ([Figure 4g](#)). Th2 cytokines, such as IL-4 and IL-6, increased with repeated challenges. Th1 cytokines, such as IFN- γ and TNF- α , decreased or tended to decrease after these challenges. IL-1 α , IL-1 β , and IL-33 were not changed significantly. IL-18 (IFN- γ inducer) and thymic stromal lymphopoietin (TSLP, a Th2-related cytokine) increased significantly. Histamine and the activity of the histamine-forming enzyme histidine decarboxylase were significantly increased after a second challenge.

Involvement of T cells and NK cells

The immune responses shown by popliteal lymph nodes to HQ are T-cell dependent ([Ewens et al., 1999](#)), whereas NK cells are reportedly involved in the CHS to haptens ([O'Leary et al., 2006](#); [Rouzaire et al., 2012](#)). Thus, we examined the roles of these cells in HQ-induced allergic ear swelling. Slight allergic ear swelling occurred after the first challenge in nude mice ([Figure 5a](#)). However, there was no augmented ear swelling after the second challenge in nude mice ([Figure 5b](#)). In severe combined immunodeficient (SCID) mice (deficient in both T and B cells), neither the first nor the second challenge produced allergic ear swelling ([Figure 5c](#) and [d](#)). Treatment of mice with an anti-asialo-GM1 antibody (an NK-cell-depleting antibody), either before sensitization or before the first challenge, did not reduce allergic ear swelling ([Figure 5e](#)). In SCID-beige mice (deficient in T, B, and NK cells), allergic ear swelling did not occur after either a first or second challenge (data not shown). However, in SCID-beige mice into which NK cells had been transferred from HQ-sensitized wild-type mice, an HQ-challenge did induce allergic ear swelling ([Figure 5f](#)). Among the various immune cells examined, macrophages and neutrophils increased slightly in nude mice but not in SCID mice (see [Supplementary Figure S3](#) online). These results suggest the existence of “memory” NK cells that are specific for HQ and that in addition to T cells, innate immunity may be important for the establishment of HQ hypersensitivity.

Involvement of IL-1

IL-1 reportedly contributes to the development of CHS ([Nambu and Nakae, 2010](#)). Indeed, allergic ear swelling was not induced in IL-1–knockout (KO) mice even after the second challenge ([Figure 5g](#) and [h](#)). In addition, in IL-1–KO mice the first and second HQ challenges did not increase

$\alpha\beta$ -T cells, $\gamma\delta$ -T cells, B cells, NK cells, macrophages, neutrophils, eosinophils, or basophils in ear pinnae (see [Supplementary Figure S3](#)), although all these cells increased or tended to increase in normal BALB/c mice sensitized to HQ. However, as shown in [Figure 4h](#), neither a first nor a second challenge with 0.5% HQ produced detectable changes in IL-1 levels in allergic ear pinnae. Thus, IL-1 may be involved in the establishment of sensitization to HQ but not in inducing allergic ear swelling. We confirmed that HQ has the ability to induce IL-1 (especially IL-1 β) when 4% HQ is painted onto mouse skin ([Figure 5i](#) and [j](#)). These results suggest that IL-1–associated innate immunity may be critically important during the step of sensitization to HQ.

Effects of anti-allergic drugs on HQ-induced allergic ear swelling

To inform possible strategies against contact hypersensitivity to HQ in humans, we thought it best to examine how we might be able to reduce the augmented allergic ear swelling after a second challenge in the present model. We noted that a second HQ challenge induces biphasic allergic ear swelling, that is, a small ear swelling peaking within 2 hours and a greater ear swelling peaking within 24–48 hours ([Figure 6a](#)). JNJ777120, dexamethasone, suplatast tosilate (Th2-cytokine inhibitor), and anti-TSLP antibody all reduced the greater ear swelling seen in the late phase, whereas pyrilamine (H1 antagonist) and cromolyn (mast cell stabilizer) reduced only the small initial ear swelling, although JNJ777120 (H4 antagonist) was effective against both phases ([Figure 6a–c](#)). These results suggest that HQ-induced CHS is of the Th2 type and that histamine H1 receptors and mast cells may not be clinically important.

DISCUSSION

C57BL/6 mice are Th1 dominant, whereas BALB/c mice are Th2 dominant ([Kelso et al., 1991](#)), and we found that HQ-induced ear swelling was induced more easily in BALB/c mice ([Figure 1](#)). It has been reported that HQ suppresses the production of IL-1 β , IFN- γ , and TNF- α in human peripheral blood monocytes *in vitro* ([Ouyang et al., 2000](#)) and of IL-12 (IFN- γ inducer) in mouse macrophages ([Kim et al., 2005](#)). [Lee et al. \(2002\)](#) found that HQ significantly increased IL-4 (Th2 cytokine) production in antigen-primed mice. Further, TSLP has been implicated in the development of CHS by promoting Th2-type responses ([Ziegler and Artis, 2010](#)). In this study, Th2 cytokines (such as IL-4 and IL-6) and TSLP were increased in HQ-induced allergic ear swelling. IFN- γ and TNF- β are known to be Th1 cytokines, and TNF- β and TNF- α share receptors. In this study, IFN- γ and TNF- α were decreased in HQ-induced allergic ear swelling. IL-18 is known to induce IFN- γ via NF- κ B ([Kojima et al., 1999](#)). In this study, IL-18 was increased in HQ-induced allergic ear swelling. It might be expected that the increased IL-18 would increase IFN- γ . However, it has been reported that HQ is a potent inhibitor of NF- κ B ([Kim et al., 2005](#); [Pyatt et al., 1998](#)), and as described, IFN- γ was decreased in the present HQ-induced allergic ear swelling. Collectively, these results indicate that HQ increases or tends to increase Th2 or Th2-related cytokines and reduce Th1 cytokines. On this

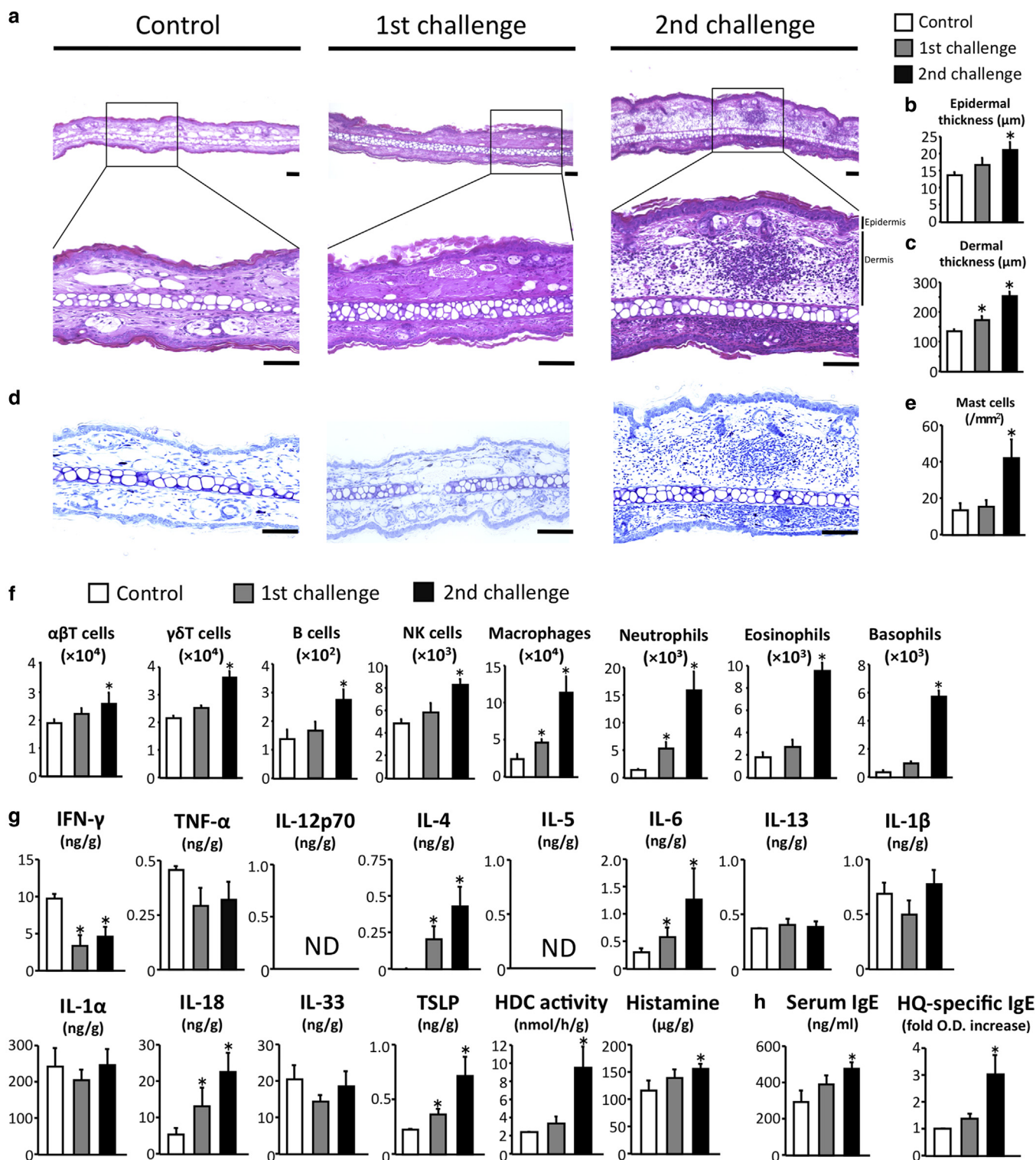


Figure 4. Inflammatory profiles of HQ-induced hypersensitivity. Mice were painted with 4% HQ on their shaved flanks. Seven days later their ear pinnas were given a first challenge and 14 days later a second challenge, each time by painting with 0.5% HQ. (a–e) Histological features. Twenty-four hours after the first and second challenges, ears were taken, fixed in 10% formalin, and embedded in paraffin. Sections were stained with (a) hematoxylin and eosin or (d) toluidine blue. Similar skin lesions were observed in a total of six animals. Quantitation of the thickness of the (b) epidermis, (c) dermis, and (e) number of mast cells in skin sections. (f) Cell infiltrations. Twenty-four hours after the first and second challenges, ears were taken and analyzed by flow cytometry (see [Supplementary Figure S2](#) for an example). (g) Cytokine profiles in HQ-induced allergic ear swelling. Mice were painted with 4% HQ on their shaved flanks. Seven days later their ear pinnas were given a first challenge and 14 days later a second challenge, each time by painting with 0.5% HQ. Twenty-four hours after the first and second challenges, ears were taken and cytokines were analyzed using ELISA kits. (h) Serum IgE at 24 hours after the first and second challenges was analyzed using an ELISA kit. In panels a–h, each value is the mean $\pm$ standard deviation from three mice. * $P < 0.01$ versus control (ear pinnas from normal mice). The results shown for a given experiment were confirmed by repeating the experiment at least once. HDC, histidine decarboxylase; HQ, hydroquinone; NK, natural killer; TNF, tumor necrosis factor; TSLP, thymic stromal lymphopoietin.

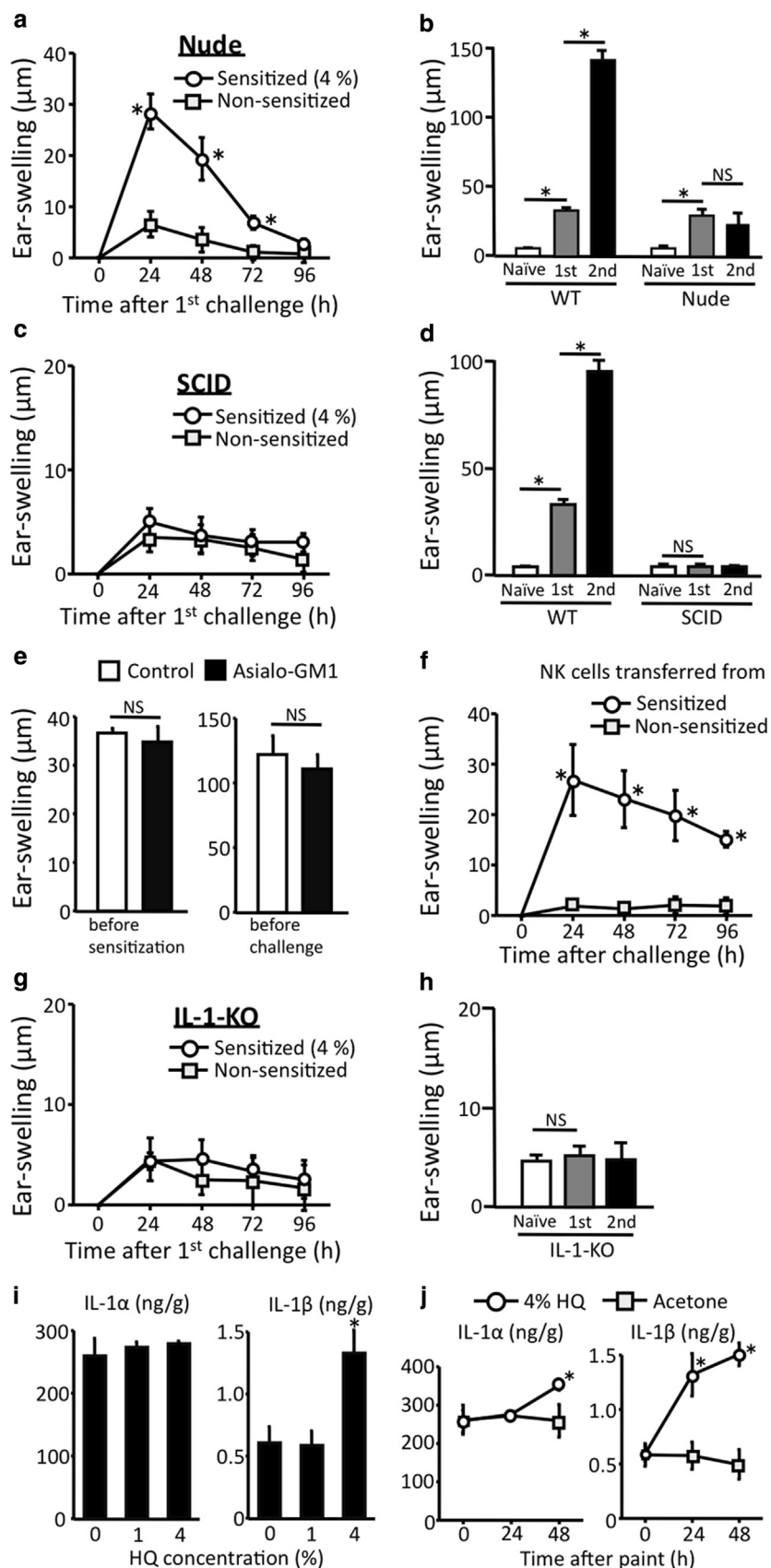
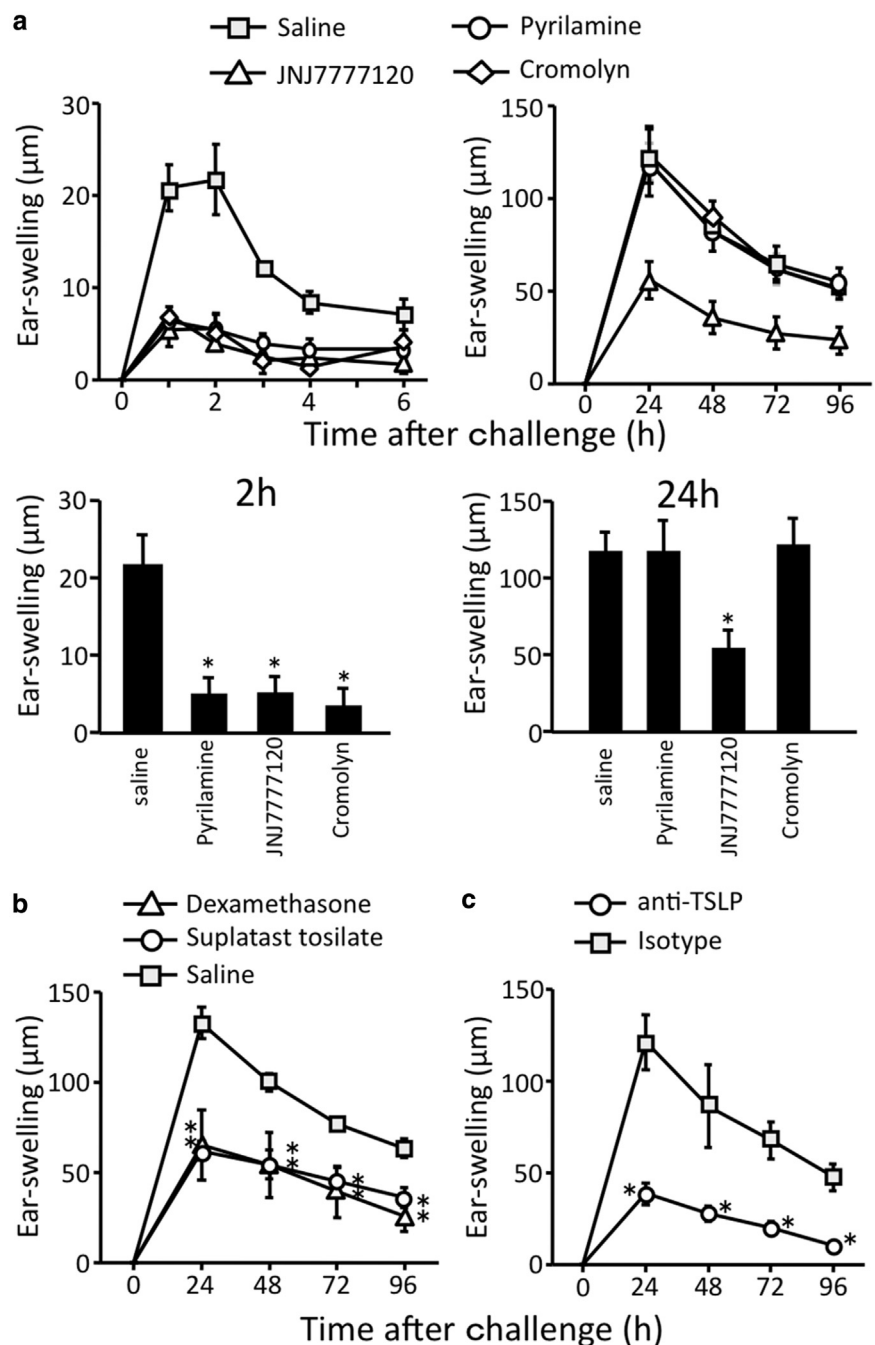


Figure 5. Characteristics of HQ-induced hypersensitivity. (a–d) Roles of T cells in HQ-induced allergic ear swelling. BALB/c WT, BALB/c nude, C.B-17 WT, and C.B-17 SCID mice were painted with 4% HQ on shaved flanks, and 7 days later their ear pinnas were challenged by painting with 0.5% HQ (first challenge). Fourteen days later, a second challenge with 0.5% HQ was delivered to the same ears. * $P < 0.01$. (e) Effects of NK-cell depletion. An anti-asialo-GM1 antibody (see [Materials and Methods](#)) was intraperitoneally injected into mice 24 hours before sensitization or 24 hours before a first HQ challenge. (f) Effects of NK-cell transfer. C.B-17 mice were painted with 4% HQ on shaved flanks. Seven days later, their ear pinnas were challenged by painting with 0.5% HQ. Five days after this challenge, liver was taken, and 1×10^5 NK cells were intravenously transferred into naïve C.B-17 SCID-beige mice. As a control, liver NK cells from naïve mice were transferred into other naïve mice. Twenty-four hours after the cell transfer, the ear pinnas of the recipients were challenged by painting with 0.5% HQ, and ear swelling was measured 24 hours after the challenge. * $P < 0.01$. (g, h) Roles of IL-1 in HQ-induced allergic ear swelling. BALB/c WT and BALB/c IL-1-KO mice were painted with 4% HQ on shaved flanks, and 7 days later their ear pinnas were challenged by painting with 0.5% HQ. Fourteen days later, a second challenge with 0.5% HQ was given to the same ears. (i, j) Effect of HQ on IL-1 in ear pinnas. In i, the indicated concentrations of HQ were painted onto ear pinnas, and 24 hours later, ears were taken for analysis of IL-1α and IL-1β. In j, 4% HQ was painted onto ear pinnas, and ears were taken at the indicated times. * $P < 0.01$ versus acetone. Throughout [Figure 5](#), each value is the mean $\pm$ standard deviation from six ears (i.e., three mice), and the results shown for a given experiment were confirmed by repeating the experiment at least once. h, hours; HQ, hydroquinone; KO, knockout; NK, natural killer; NS, not significant; SCID, severe combined immunodeficient; WT, wild type.

Figure 6. Effects of therapeutic agents on HQ-induced allergic ear swelling. Mice were painted with 4% HQ on their shaved flanks. Seven days later their ear pinnae were given a first challenge and 14 days later a second challenge, each time by painting with 0.5% HQ. Test reagents were intraperitoneally injected two times: 1 hour before and 1 hour after the second challenge. (a) Effects of saline, pyrilamine (50 mg/kg), cromolyn (50 mg/kg), and JNJ777120 (50 mg/kg). * $P < 0.01$ versus saline. (b) Effects of saline, dexamethasone (10 mg/kg), and suplatast tosilate (50 mg/kg). * $P < 0.01$ versus saline. (c) Effects of anti-TSLP antibody (20 μ g) and Rat IgG1 (20 μ g) isotype (control). * $P < 0.01$ versus isotype control. Each value is the mean $\pm$ standard deviation from six ears (i.e., three mice). The results shown for a given experiment were confirmed by repeating the experiment at least once. h, hours; HQ, hydroquinone; TSLP, thymic stromal lymphopoietin.



basis, HQ-induced allergic ear swelling should be characterized as a Th2-type hypersensitivity.

OX and fluoro- or chlorobenzene derivatives having di- or trinitro substituents are typical haptens that induce Th1-type hypersensitivity (Hayashi et al., 2001), and trimellitic anhydride and FITC are typical haptens that induce Th2-type hypersensitivity (Imai et al., 2006; Hayashi et al., 2001; Kitagaki et al., 1997; Schneider et al., 2009). However, repeated challenges with Th1 haptens reportedly induce a shift to Th2-type responses (Kitagaki et al., 1997). FITC cannot by itself induce hypersensitivity in mice, and a phthalate ester, such as dibutyl phthalate (used as a solvent), is needed as an adjuvant (Imai et al., 2006). Shigeno et al. (2009) found that

dibutyl phthalate stimulates the production of TSLP, and TSLP mediates the hypersensitivity to FITC. Unlike FITC, HQ by itself can induce Th2-type hypersensitivity, as shown here. We found that HQ was capable of inducing TSLP, and this induction was augmented by repeated HQ challenges. These results suggest that (i) HQ-induced Th2-type hypersensitivity may be mediated by TSLP and (ii) TSLP might also be important in the ability of repeated challenges to augment Th2-type responses or in the shift from Th1- to Th2-type hypersensitivity.

NK cells have been shown to be involved in CHS to such haptens as dinitrofluorobenzene. However, the contribution of NK cells to CHS is controversial. Indeed, although the

adoptive cell transfer experiments of O'Leary et al. (2006) indicated that NK cells are both necessary and sufficient to induce CHS in recipient Rag2^{-/-}Il2rg^{-/-} mice, Rouzaire et al. (2012) indicated that the NK-cell-mediated CHS induced in recipient CD3ε^{-/-} mice is much smaller than the T-cell-mediated CHS. In this study, we found that (i) no detectable allergic ear swelling occurred in IL-1-KO mice and (ii) HQ did induce allergic ear swelling in C.B-17 SCID-beige mice that had received an adoptive transfer of NK cells. These findings suggest that (i) IL-1-associated innate immunity might be important at the step at which sensitization occurs to HQ and (ii) T-cell-dependent acquired immunity and NK-cell-dependent innate immunity are both important during CHS to HQ. Our future studies will aim to clarify the detailed molecular and cellular mechanisms underlying Th2-type hypersensitivity to HQ, including the role of IL-1 in the step of sensitization to HQ and the mechanisms by which a second challenge augments CHS to HQ.

We showed here that JNJ7777120 (H4R antagonist), dexamethasone (anti-inflammatory steroid), suplatast tosilate (Th2-cytokine inhibitor), and anti-TSLP antibody all reduced the allergic ear swelling seen after a second challenge, whereas pyrilamine (H1R antagonist) and cromolyn (mast-cell stabilizer) reduced only the transient, weak ear swelling seen in the early period after a second challenge. We also found that histamine and histidine decarboxylase activity were significantly increased in the HQ-induced ear swelling occurring after a second challenge. Cowden et al. (2010) reported that an H4R antagonist reduced Th2-type inflammation in mice, and Tamaoki et al. (2000) noted that suplatast tosilate improves asthma. These results suggest that Th2-cytokine inhibitors, anti-inflammatory steroids, and H4R antagonists may have therapeutic potential against HQ hypersensitivity.

It has been reported that inflammatory substances exert adjuvant effects in the establishment of the CHS induced by organic haptens (Grabbe et al., 1996). In practice, HQ is often used in combination with other chemicals. For instance, tretinoin plus HQ is used in cosmetics for reducing wrinkles. Tretinoin is known to induce irritant inflammation and has been reported to activate the TRPV1 receptor (Yin et al., 2013). TRPV1 is reportedly involved in the adjuvant effects of CHS (Shiba et al., 2009). Here, we found that tretinoin acts as an adjuvant in the induction of hypersensitivity to HQ. In dentistry, HQ is used to prevent the polymerization of resin monomers. Although resin monomers, when intradermally injected, are inflammatory (Leggat et al., 2003), they have very little, if any, potential as haptens in mice (Bando et al., 2014). Instead, resin monomers exhibit potent adjuvant effects in the induction of allergic responses to nickel, one of the most widely distributed metals in our environment (Bando et al., 2014). Here, we found that resin monomers exhibit adjuvant effects in HQ-induced hypersensitivity, too. Thus, what are thought of as examples of hypersensitivity to resin monomers might actually be hypersensitivity to HQ, nickel, or other haptens. In any event, caution should be exercised when combining HQ with other inflammatory chemicals.

In conclusion, HQ is a potent hapten in the induction of Th2-type hypersensitivity in mice, which is established

by innate immunity and acquired immunity. HQ-hypersensitivity is promoted by other inflammatory chemicals.

MATERIALS AND METHODS

Animals

BALB/c, C57BL/6N, and BALB/c nude mice were purchased from SLC (Shizuoka, Japan). C.B-17, C.B-17 SCID, and C.B-17 SCID-beige mice were purchased from Charles River Japan (Yokohama, Japan). IL-1-KO BALB/c mice were established from original IL-1α-KO BALB/c and IL-1β-KO BALB/c mice (Horai et al., 1998).

Reagents

HQ, OX (4-ethoxymethylene-2-phenyl-2-oxazolin-5-one), tretinoin, MMA, HEMA, isoeugenol, dexamethasone, JNJ7777120, cromolyn, pyrilamine, and suplatast tosilate were purchased from Sigma-Aldrich (St. Louis, MO). All other reagents were from Wako Pure Chemical Ind., Ltd. (Osaka, Japan), unless otherwise indicated. HQ, alpha-hexylcinnamaldehyde, isoeugenol, tretinoin, MMA, OX, and HEMA were dissolved in acetone. Dexamethasone, pyrilamine, cromolyn, and suplatast tosilate were dissolved in saline. JNJ7777120 was dissolved in dimethyl sulfoxide and diluted with phosphate buffered saline.

Local lymph node assay

LLNA was performed as described by Takeyoshi et al. (2005). Briefly, 25 μl of a test solution was painted onto ear pinnae daily for three consecutive days. A single intraperitoneal injection of BrdU (5 mg/mouse) was given on day 4. The auricular lymph nodes were removed on day 5. The level of BrdU incorporation was measured by means of an ELISA kit. EC3 was calculated as described by Basketter et al. (2007).

Sensitization to HQ and elicitation of allergic inflammation

Mice were sensitized to HQ by painting an acetone solution of HQ onto shaved flanks (100 μl/mouse). Seven days later, ear pinnae were challenged by painting an acetone solution of HQ (10 μl/each side of the ear). In some experiments, a second challenge (10 μl/each side of the ear) was performed on day 14. Ear swelling was measured at the indicated times using a Peacock dial thickness gauge (Ozaki MFG Co. Ltd, Tokyo, Japan), with the induced swelling being calculated as the difference in thickness between before and after the challenge. Detailed protocols are given in the text or in the legends to the figures relating to each experiment.

Sensitization to OX and elicitation of OX allergic inflammation

Mice were sensitized to OX by painting a 4% acetone solution of OX onto shaved flanks (100 μl/mouse). Seven days later, ear pinnae were challenged by painting a 0.5% acetone solution of OX (10 μl/each side of the ear).

Adoptive transfer of spleen cells in BALB/c mice

BALB/c mice were sensitized to HQ, and 7 days later they were challenged with HQ (to expand immune cells) as described. Two days after the challenge, the spleen was collected, and 1 × 10⁷ spleen cells were intravenously transferred into a nonsensitized BALB/c mouse. One day after this transfer, the recipient's ears were challenged with 0.5% HQ.

Depletion of NK cells

Rabbit serum containing anti-asialo-GM1 antibody (100 μl) was dissolved in saline (1 ml), and this solution was intraperitoneally injected into mice (0.2 ml/mouse).

Adoptive transfer of liver NK cells

C.B-17 mice were sensitized to HQ, and 7 days later they were challenged with HQ (to expand immune cells) as described above. Five days after the challenge, the liver was collected and NK cells were isolated essentially as described previously (Rouzaire et al., 2012). Then, 1×10^5 liver NK cells were intravenously transferred into a nonsensitized C.B-17 SCID-beige mouse. One day after this transfer, the recipient's ears were challenged with 0.5% HQ.

Measurements of cytokines and histamine in ear pinnas and of serum IgE

Cytokines in ear pinnas were measured as described previously (Deng et al., 2006, 2012) using ELISA kits for IL-1 α , IL-1 β , IFN- γ , and TNF- α (Thermo Scientific, Waltham, MA); IL-5, IL-12p70, IL-13 and IL-33, and TSLP (Bioscience, San Diego, CA); IL-4 and IL-6 and IgE (BioLegend, San Diego, CA); and IL-18 (MBL, Nagoya, Japan). Histamine and histidine decarboxylase activities in ear pinnas were determined as described previously (Endo et al., 1983; Wu et al., 2004).

Histology

At 24 hours after challenge with HQ, ear pinnas were fixed in 10% formalin and embedded in paraffin. Sections (6- μ m thick) were stained with hematoxylin and eosin or with toluidine blue.

Flow cytometry

Twenty-four hours after challenge with HQ, ear pinnas were cut into small pieces and incubated with RPMI1640 containing 10% fetal bovine serum and 1 mg/ml collagenase IV (Sigma-Aldrich) for 2 hours at 37 °C in a shaking water bath. The digested tissues were washed with phosphate buffered saline and passed through a 70- μ m cell strainer. The cells were incubated with 10 μ g/ml Fc block (clone 2.4G2) for 15 minutes on ice. They were then stained with Pacific blue-labeled anti-CD45 antibody, APC/Cy7-labeled and PE-labeled anti-IgE antibody, APC-labeled anti-CD49b antibody, PerCP/Cy5.5-labeled anti-TCR β antibody, FITC-labeled anti-TCR $\gamma\delta$ antibody, PE/Cy7-labeled anti-CD19 antibody, PerCP/Cy5.5-labeled anti-Ly6G antibody, PE/Cy7-labeled anti-CD11c antibody, PE-labeled siglec F antibody, APC/Cy7-labeled anti-CD11b antibody, or PE-labeled Anti-CD3 ϵ antibody (BioLegend) for 30 minutes on ice. Dead cells were stained with DAPI and excluded from further analysis. The antibody-stained cells were analyzed with the aid of LSRFortessa (BD Biosciences, San Jose, CA), and data were analyzed using FlowJo software (Tree Star, Ashland, OR). In this study, flow cytometry was performed on spleen, too, for confirmatory immunostaining. Gating strategy is depicted in Supplementary Figure S4.

Statistical analysis

Experimental values are given as the mean $\pm$ standard deviation. The statistical significance of differences was analyzed using a Bonferroni multiple comparison test after analysis of variance with the aid of InStat software (InStat, Scottsdale, AZ). A *P*-value less than 0.01 was considered to indicate significance.

Study approval

All experimental protocols were approved by the Institutional Animal Care and Use Committee and by the Genetic Modification Safety Committee of Tohoku University (Sendai, Japan). All experiments complied with Regulations for Animal Experiments and Related Activities at Tohoku University (Sendai, Japan).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at <http://dx.doi.org/10.1016/j.jid.2016.12.018>.

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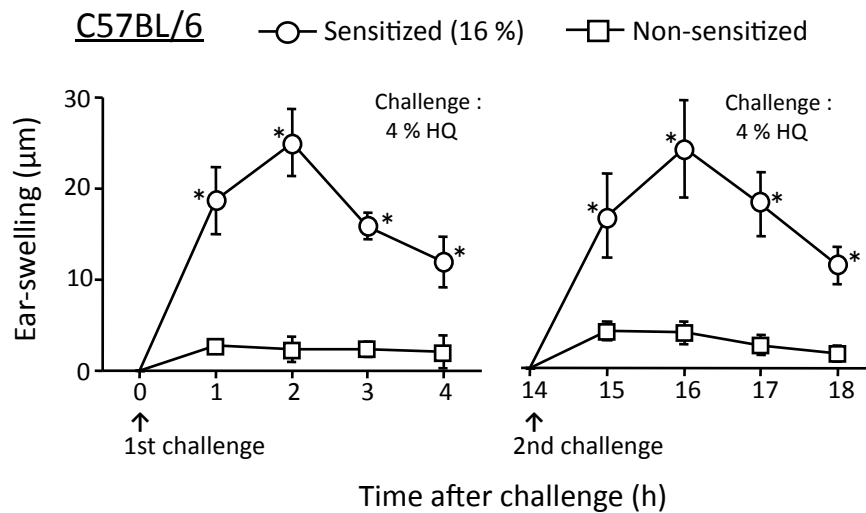


Figure S1. Effects of repeated challenges with HQ in C57BL/6 mice.

Shaved flanks were painted with 16 % HQ, and 7 days later ear-pinnas were challenged with 4% HQ (1st challenge). Fourteen days later, a 2nd challenge with 4% HQ was given to the same mice. * $P < 0.01$ vs. non-sensitized naïve mice at the same time-point. The results shown for a given experiment were confirmed by repeating the experiment at least once.

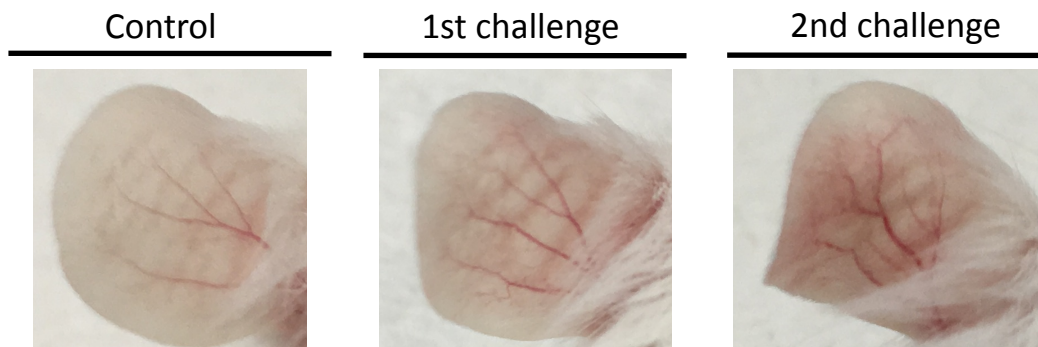


Figure S2. Photographs of ear-pinna skins.

Mice were painted with 4 % HQ on their shaved flanks. Seven days later their ear-pinnas were given a 1st challenge and 14 days later a 2nd challenge, each time by painting with 0.5 % HQ. Photographs of ear-pinna skins 24 h after the 1st and 2nd challenges. Similar lesions were observed in a total of 6 animals.

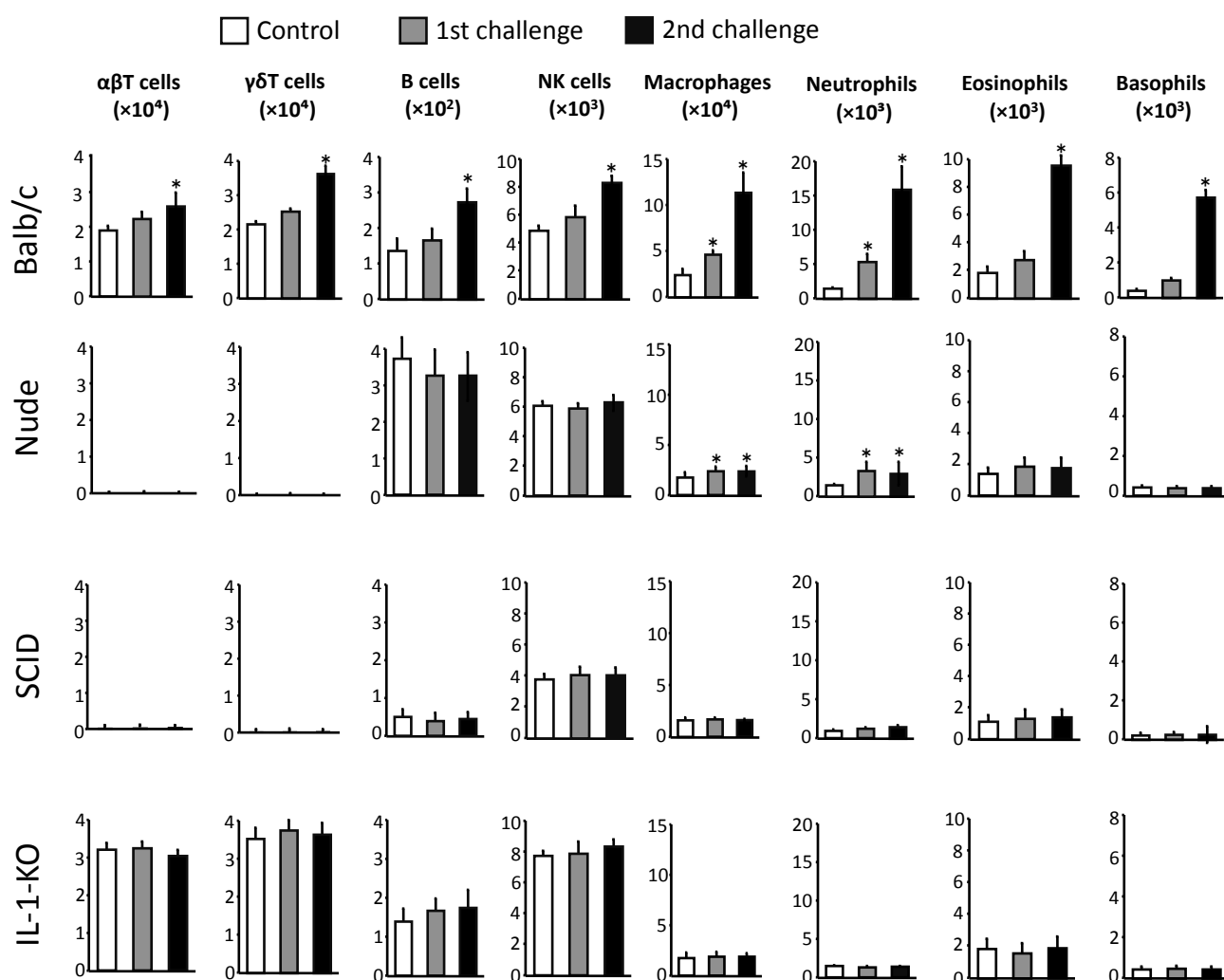


Figure S3. Cell infiltrations in ear-pinnas in BALB/c, nude, SCID, and IL-1-KO mice.

Twenty-four hours after the 1st and 2nd challenges, ears were taken and analyzed by flow cytometry (see Materials and Methods). Each value is the mean $\pm$ SD from 6 ears (i.e., 3 mice). * $P < 0.01$ vs. control (ear-pinnas from non-sensitized mice). The results shown for a given experiment were confirmed by repeating the experiment at least once.

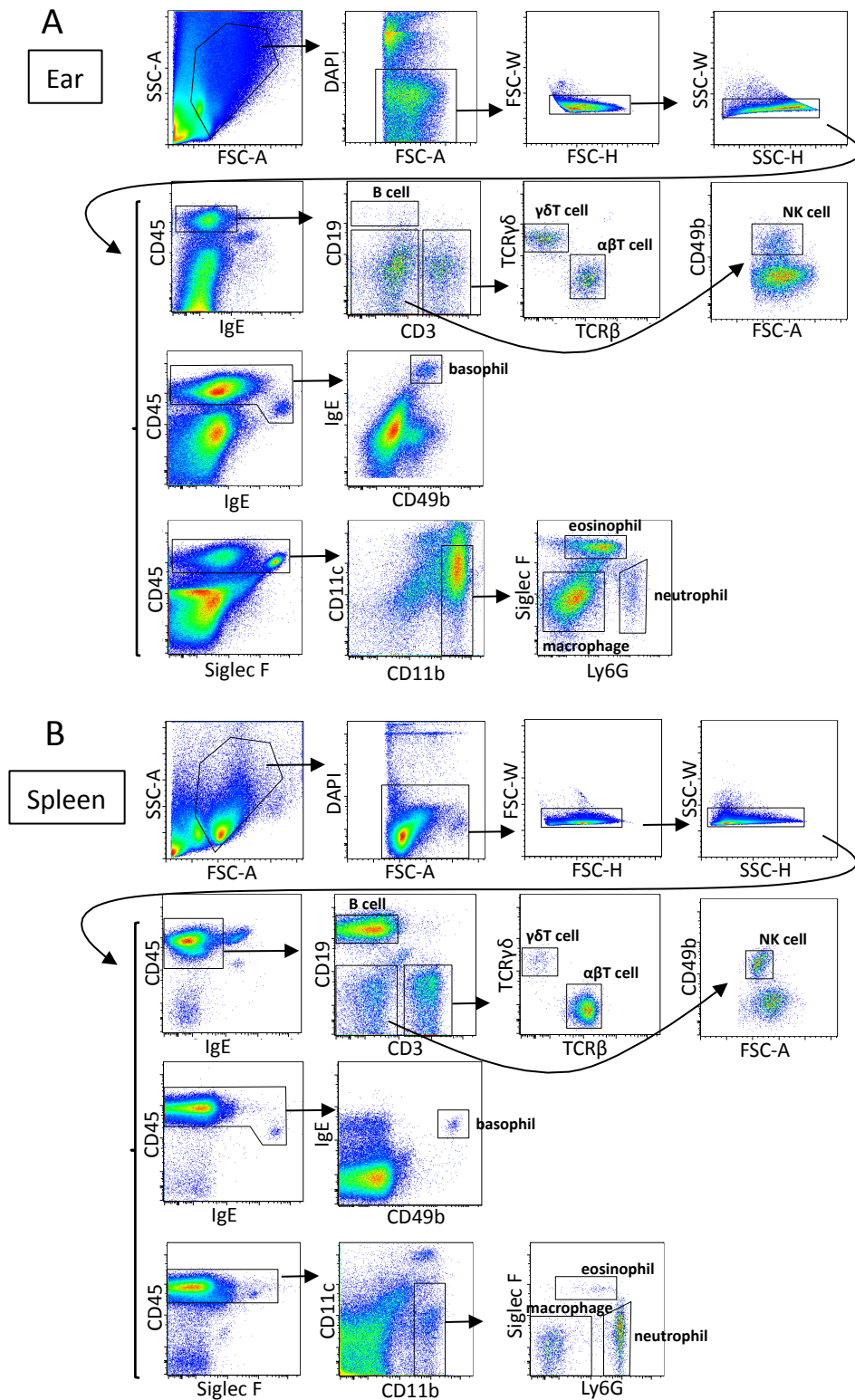


Figure S4. Flow cytometry gating strategy.

Cells prepared from ears (a) and spleens (b) of normal mice (see Materials and Methods) were dispersed and stained with CD45, CD3, CD19, TCR β , TCR $\gamma\delta$, CD49b, IgE, Siglec F, CD11b, CD11c, or Ly6G monoclonal antibodies to identify various cell subsets.