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Low Zone Tolerance Requires ICAM-1 Expression to Limit Contact Hypersensitivity Elicitation

Running title: Low dose tolerization abrogates ICAM-1 up-regulation

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Abstract

Painting subsensitizing doses of contact sensitizers on skin (low dose tolerization) induces antigen-specific tolerance, known as low zone tolerance (LZT), which has been experimentally demonstrated by inhibition of contact hypersensitivity (CHS). Although LZT was resulted from inhibition of the sensitization phase, the effects on the effector/elicitation phase remain unknown. L-selectin and ICAM-1 regulate leukocyte influx into inflamed tissues during the elicitation phase of CHS. LZT was investigated in mice lacking either L-selectin or ICAM-1 to evaluate the roles these leukocyte receptors playing in LZT during the elicitation phase. Low dose tolerization effectively suppressed CHS in wild type and L-selectin-deficient mice, but not in ICAM-1-deficient mice. Low dose tolerized ICAM-1-deficient splenocytes effectively suppressed the elicitation phase in naïve wild type recipients. Sensitized ICAM-1-deficient splenocytes exhibited normal proliferative responses to the sensitizing antigen and generated normal CHS in wild type recipients. Thus, ICAM-1 deficiency did not affect the sensitization. LZT associated with a lack of ICAM-1 up-regulation after elicitation, suggesting a potentially mechanistic role for ICAM-1. Blockade of IL-10, a possible mediator of LZT, produced by hapten-specific suppressor cells, abrogated LZT and restored ICAM-1 up-regulation. These results indicate that low dose tolerization controls CHS by abrogating ICAM-1 up-regulation during the elicitation phase.

Introduction

There are several established animal models of acquired peripheral tolerance in which mature lymphocytes in peripheral lymphoid tissues are rendered nonfunctional or hyporesponsive to specific antigens. Ultraviolet (UV) exposure or low, subimmunogenic doses of antigen are two ways to induce antigen-specific peripheral tolerance (Maurer *et al.*, 2003; Ullrich, 1995). When antigen is repeatedly applied epicutaneously, low zone tolerance (LZT) is established. Because LZT is achieved by the physiological uptake of very small amounts of allergenic antigens through skin (low dose tolerization), it is widely regarded as a highly relevant model of naturally occurring tolerance to contact immunogens. Transfer of lymphoid components from tolerized donors induces LZT in naive recipients (Maurer *et al.*, 2003). Therefore, one of the possible mechanisms of LZT is the generation of antigen-specific suppressor cells, which would inhibit the generation and activation of effector T cells.

LZT is demonstrated by the inhibition of cellular immune responses, such as contact hypersensitivity (CHS). CHS consists of two phases, the sensitization phase and the elicitation phase. Although much is known about the sensitization phase, little is known about the elicitation phase (Grabbe *et al.*, 1996). Similarly, while the suppressor T cells generated in low dose tolerization can suppress the sensitization phase by an IL-10-dependent pathway, the effect of these cells on the elicitation phase remains unknown (Maurer *et al.*, 2003). Recent findings have demonstrated that the elicitation phase of CHS is regulated by various factors including cytokines and inflammatory cells at the sites of elicitation (Grabbe and Schwarz, 1998). Resident skin cells, mainly keratinocytes, are likely to initiate the elicitation phase of CHS responses by producing proinflammatory cytokines (Grabbe and Schwarz, 1998;

Piguet *et al.*, 1991). A stimulatory role is attributed primarily to tumor necrosis factor (TNF)-α, since intradermal injection of this cytokine results in CHS-like skin inflammation (Grabbe and Schwarz, 1998; Granstein *et al.*, 1986; Piguet *et al.*, 1991). Expression of cell adhesion molecules also regulates the capacity to elicit CHS responses, since leukocyte recruitment into inflamed sites is achieved using specific constitutive or inducible adhesion molecules (Butcher, 1991; Fujimoto *et al.*, 1999; Grabbe and Schwarz, 1998; Springer, 1995). L-selectin is constitutively expressed by most leukocytes, while P-selectin and E-selectin are expressed by activated endothelial cells (Tedder *et al.*, 1999). Interaction with its ligand of N-glycan-linked 6-sulfo sialyl Lewis X, L-selectin mediates leukocyte capture and rolling on the endothelium (Mitoma *et al.*, 2007; Tedder *et al.*, 1999). L-selectin-deficient (L-selectin$^{-/-}$) mice consistently exhibit decreased CHS responses (Staite *et al.*, 1996; Steeber *et al.*, 1999). ICAM-1 is constitutively expressed by endothelial cells and is rapidly up-regulated by the local release of proinflammatory cytokines, including TNF-α (Butcher, 1991; Springer, 1995). The interaction of ICAM-1 with β_2 integrins on leukocytes enhances rolling, firm adhesion, and transmigration of leukocytes at sites of inflammation (Butcher, 1991; Springer, 1995; Tedder *et al.*, 1999). Furthermore, ICAM-1 deficiency inhibits the elicitation phase of CHS responses without affecting the sensitization phase (Steeber *et al.*, 1999).

In the current study, we investigated the roles of low dose tolerization-induced suppressor cells on the elicitation phase of CHS responses. We found that low dose tolerization-induced suppressor cells affected the elicitation phase of CHS by inhibiting the ICAM-1 up-regulation in inflamed tissues.

Results

Low dose tolerization inhibits ICAM-1 up-regulation after antigen challenge

The elicitation phase of CHS is dependent upon inflammatory cell infiltration, which is tightly regulated by cell adhesion molecules, including ICAM-1. ICAM-1 is constitutively expressed on vascular endothelial cells and the expression is strongly up-regulated during the elicitation phase of CHS (McHale *et al.*, 1999). To address the role of ICAM-1 during LZT, we first examined ICAM-1 mRNA production following antigen challenge in mice that had either undergone low dose tolerization and sensitization, sensitization only, or challenge only. For low dose tolerization, mice were painted 5 times with tolerizing doses of 2,4,6-trinitro-1-chlorobenzene (TNCB) onto back skin. Then, mice were sensitized by TNCB onto the back skin. Five days later, ears were elicited. Total RNA samples, collected from ears taken 12 hours after the elicitation of CHS, were used for semi-quantitative real-time PCR analysis of ICAM-1 expression (Figure 1). CHS induced a greater than ten-fold up-regulation of ICAM-1 production when compared with elicitation without sensitization (P < 0.05). This up-regulation was diminished in mice that had undergone low dose tolerization before sensitization. Thus, low dose tolerization abrogated ICAM-1 up-regulation at the elicitation site.

Low dose tolerization does not suppress CHS in ICAM-1$^{-/-}$ mice

For further analysis of the effects of low dose tolerization on adhesion molecules, LZT was assessed in ICAM-1$^{-/-}$ mice, which can not express or up-regulate ICAM-1, L-selectin$^{-/-}$ mice, and double knockout ICAM-1/L-selectin$^{-/-}$ mice. Mice were sensitized by TNCB onto the back skin after low dose tolerization. Five days later, ears were challenged and ear swelling was measured after 48 hours. In wild type mice, low dose tolerization inhibited the elicitation phase (swelling decreased by 55%, P <

0.0001; Figure 2). Histopathology of the ear sections showed that inhibition of ear swelling was associated with a reduction in both oedema and leukocyte infiltration (data not shown). CHS responses were reduced in L-selectin$^{-/-}$ mice (54% decrease, P < 0.001), ICAM-1$^{-/-}$ mice (48%, P < 0.005), and L-selectin/ICAM-1$^{-/-}$ mice (85%, p < 0.0001), relative to wild type mice (Figure 2). Low dose tolerization significantly suppressed CHS responses in L-selectin$^{-/-}$ mice (68% decrease, P < 0.001) compared to L-selectin$^{-/-}$ mice without tolerization. By contrast, ICAM-1$^{-/-}$ mice did not exhibit LZT (Figure 2). Interestingly, low dose tolerization suppressed CHS responses in wild type mice to a similar level to that observed in ICAM-1$^{-/-}$ mice that had not undergone tolerization. Thus, these results suggest that LZT is dependent on ICAM-1 expression.

ICAM-1 deficiency does not affect antigen-specific proliferative responses

Since ICAM-1 can provide costimulatory signals for T cell activation during sensitization, we assessed whether the loss of LZT in ICAM-1$^{-/-}$ mice resulted from impaired antigen presentation, and generation of antigen-specific effector cells. Antigen-specific proliferative responses to 2,4,6-trinitro-benzenesulfonic acid (TNBS, a water soluble analog of TNCB) were analyzed. Five days after sensitization, splenocytes were isolated and cultured in vitro with TNBS for 5 days. In sensitized animals, similar splenocyte proliferation was observed at 20mM TNCB in both ICAM-1$^{-/-}$ mice and wild type mice, when compared with unsensitized mice (P < 0.03 and P < 0.01, respectively, Figure 3). Low dose tolerization did not affect proliferation in sensitized ICAM-1$^{-/-}$ (P = 0.14) or wild type mice (P = 0.26) at 20mM TNCB. For further analysis, antigen-specific proliferative responses TNBS were analyzed using lymph node cells, since these cells may include high number of immunized T cells. Low dose tolerization did not affect proliferation in sensitized

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ICAM-1$^{-/-}$ or wild type mice. In addition, the response was significantly decreased in CD8$^+$ depleted lymph node cells in both ICAM-1$^{-/-}$ (P < 0.03) and wild type mice (P < 0.05), suggesting that CD4$^+$ cells may suppress the antigen specific proliferative response. Thus, ICAM-1 deficiency did not affect the generation of antigen-specific effector cells.

ICAM-1 is not required for the generation and function of suppressor T cells

Low dose tolerization generates T suppressor cells that can be transferred to naive mice by adoptive transfer of splenocytes (Seidel-Guyenot *et al.*, 2006). We assessed the effect of ICAM-1 deficiency on the generation and function of these suppressor cells using adoptive transfer experiments. Seven days after TNCB sensitization following low dose tolerization, splenocytes from donor mice were transferred into naive recipients that were subsequently sensitized by TNCB. After 5 days, recipient mice were challenged on the ear and ear thickness was measured 48 hours later. Wild type mice that had received splenocytes from sensitized wild type or sensitized ICAM-1$^{-/-}$ donors exhibited normal CHS responses (Figure 4A). The transfer of splenocytes from low dose tolerized wild type mice into wild type recipients resulted in a significantly reduced CHS response compared to wild type recipients that received splenocytes from sensitized wild type mice (by 59%, p < 0.001). However, this reduction was completely abrogated by CD8$^+$ cell depletion from donor cells. Similarly, adoptively transferred spleen cells from tolerized ICAM-1$^{-/-}$ donors suppressed the CHS response in wild type recipients (by 61%, P < 0.001). Thus, the generation and function of suppressor activity did not require ICAM-1 expression. By contrast, the transfer of splenocytes from low dose tolerized wild type donors into ICAM-1$^{-/-}$ recipients did not suppress the CHS response (Figure 4B). These results suggest that LZT requires ICAM-1 expression in recipients.

Suppressor cells inhibit effector cells during the elicitation phase of CHS

Previous studies have shown that UV-induced suppressor cells can attenuate the elicitation phase of CHS as well as the sensitization phase (Komura *et al.*, 2003). To assess the function of low dose tolerization-induced suppressor cells on the elicitation phase, we conducted further adoptive transfer studies. Naive wild type recipients received intravenously a mixture of spleen cells from sensitized wild type donors containing effector cells and the same number of spleen cells from low dose tolerized wild type donors containing antigen-specific suppressor cells. After 5days, the ears of unsensitized recipients were challenged and the ear swelling responses were evaluated. A normal CHS response was induced by the transfer of splenocytes from sensitized wild type donors into naive wild type recipients (Figure 5A). Co-transfer of splenocytes from low dose tolerized wild type donors along with splenocytes from sensitized wild type donors significantly suppressed CHS responses compared to wild type recipients of splenocytes from sensitized donors (by 61%, P < 0.005). Therefore, tolerized suppressor cells can inhibit the effector cells during the elicitation phase of the CHS response. By contrast, co-transfer of splenocytes from sensitized wild type donors along with those from low dose tolerized wild type donors into ICAM-1$^{-/-}$ recipients did not result in further suppression of the response when compared to the transfer of cells only from sensitized wild type donors (Figure 5B). These results suggest that LZT requires ICAM-1 expression in recipients.

Anti-IL-10 mAb treatment during the elicitation phase restores LZT

Keratinocytes are one of the major sources of TNF-α, and IL-10 down-regulates TNF-α production by keratinocytes (McHale *et al.*, 1999; Piguet *et al.*, 1991). Keratinocyte-produced TNF-α augments endothelial ICAM-1 expression (McHale *et al.*, 1999; Piguet *et al.*, 1991). In addition, IL-10 is involved in mediating tolerance

induced by LZT (Maurer *et al.*, 2003). Therefore, it is possible that low dose tolerization-induced suppressor cells augment local IL-10 release, which is responsible for the decreased TNF-α production, resulting in a lack of ICAM-1 up-regulation. To assess this possibility, the effect of IL-10 blockade on LZT was examined.   We injected anti-IL-10 mAbs immediately before antigen challenge in sensitized mice. First, TNF-α production 12 hours after challenge was significantly suppressed in low dose tolerized wild type mice as well as in low dose tolerized ICAM-1$^{-/-}$ recipients, when compared to CHS responses for each genotype (P < 0.03 and P < 0.05, respectively, Figure 6a). Intradermal anti-IL-10 mAb injection in tolerized wild type mice restored CHS responses to the level observed in normal wild type CHS responses. Nonetheless, this restoration by IL-10 blockade was not observed in ICAM1$^{-/-}$ mice (Figure 6b). In addition, the restoration of LZT by anti-IL-10 mAbs was associated with up-regulation of TNF-α mRNA (Figure 6c) and ICAM-1 mRNA (Figure 6d) following antigen challenge. Thus, IL-10 blockade during the elicitation phase eliminated LZT by allowing for the up-regulation of TNF-α and ICAM-1.

Discussion

In the current study, low dose tolerization effectively suppressed the elicitation phase of CHS, which we found to be dependent upon suppressor cells blocking ICAM-1 up-regulation. In fact, ICAM-1 up-regulation at the challenge sites was abrogated in low dose tolerized wild type mice (Figure 1). LZT was found to be lacking in ICAM-1^{-/-} mice, although they could sufficiently generate antigen-specific effector cells (Figure 3 and 4). Adoptive transfer studies further indicated that tolerized cells inhibited the effector cells during the elicitation phase by an ICAM-1-dependent mechanism (Figures 5). These findings indicate that LZT associates with abrogation of ICAM-1 up-regulation at the challenge site.

Previous studies have revealed that ICAM-1 is highly expressed on endothelium within the skin (Komura *et al.*, 2003; McHale *et al.*, 1999). Therefore, the major source of ICAM-1 mRNA in the current study is likely to be endothelial cells. Although several cell types, including keratinocytes, also express ICAM-1 and up-regulate ICAM-1 expression during CHS responses (Albanesi *et al.*, 1999; von den Driesch *et al.*, 1995). Further studies, such as immunohistchemistry for ICAM-1 during the elicitation phase, are needed to clarify the issue. Adoptive transfer experiments into ICAM-1^{-/-} bone marrow chimeras will be revealing. In the present study, enhanced TNF-α production following antigen challenge was eliminated by low dose tolerization in ICAM-1^{-/-} mice as well as wild type mice (Figure 6). Furthermore, blockade of IL-10 abrogated LZT, which was associated with restored TNF-α and ICAM-1 up-regulation following antigen challenge (Figure 6). Collectively, these results indicate that a lack of increased TNF-α production following antigen challenge is responsible for a lack of endothelial ICAM-1 up-regulation in LZT.

The results of the present study suggest that ICAM-1 up-regulation critically controls CHS responses, although ICAM-1$^{-/-}$ mice generated a certain level of CHS responses (Figure 2). The residual CHS responses in ICAM-1$^{-/-}$ mice is likely to be regulated by other adhesion molecules including L-selectin, since genetic loss of both ICAM-1 and L-selectin inhibited the majority of CHS responses with significant reduction of leukocyte infiltration into the skin (Figure 2) (Komura *et al.*, 2003; Steeber *et al.*, 1999). It was also noted that low dose tolerization suppressed wild type CHS responses to CHS levels in L-selectin$^{-/-}$ mice, which were completely suppressed by low dose tolerization. These observations are also consistent with the current conclusion that low dose tolerization suppressed ICAM-1 up-regulation without affecting the expression of other cell adhesion molecules, including L-selectin. Additionally, a role of ICAM-1 in CHS is well-established during leukocyte rolling, firm adhesion, and transmigration of leukocytes into sites of inflammation (Argenbright *et al.*, 1991). Thus, ICAM-1 expression levels are closely linked to the CHS response.

Humans generate adaptive immune systems during growth and development, which can effectively eliminate pathogens while avoiding immune responses directed against self antigens. UV exposure and low dose tolerization help to establish normal peripheral tolerance to contact allergens and avoid excessive immune responses (Schwarz, 2005). Recently, it has been suggested that UV-induced CD4$^+$CD25$^+$ regulatory T cells are the major mediator of UV-induced tolerance, since tolerance was transferable to naive recipients by this T cell subset (Aragane *et al.*, 2003; Schwarz *et al.*, 2007). However, CD8$^+$ T cells plays a role to suppress the CHS in adoptive transfer studies in the present study and the previous work (Steinbrink *et al.*, 1996). Since CD4$^+$ cells also suppressed the antigen specific proliferative

response after the sensitization (Figure 3b), LZT may be coordinately regulated by several immune subsets, including $CD4^+$ and $CD8^+$ cells. Multiple studies at various phase of CHS may add the information about the mediator of LZT in the future. The suppressor cells may suppress the elicitation phase of CHS in an ICAM-1- and IL-10-dependent manner in both the LZT model utilized in the current study as well as UV-induced tolerance models (Komura *et al.*, 2003). However, the relationship between suppressor cells and ICAM-1 and IL-10 is not clear in this study. Since the elicitation phase, but not the sensitization phase, is crucial for LZT-induced suppression of CHS, further elucidating the mechanisms of peripheral tolerance regulating the elicitation phase will direct clinical approaches for the management of imbalanced adaptive immune systems, including autoimmune and allergic diseases.

Materials and Methods

Mice

ICAM-1$^{-/-}$ mice were obtained from The Jackson Laboratory (Bar Harbor, ME) (Sligh Jr. *et al.*, 1993). L-selectin$^{-/-}$ mice and mice lacking both L-selectin and ICAM-1 (L-selectin/ICAM-1$^{-/-}$) were generated as described previously (Arbones *et al.*, 1994). All mice were healthy, fertile, and did not display evidence of infection or disease. All mice were backcrossed between 5 to 10 generations onto the C57BL/6 genetic background. Mice used for experiments were 12 to 16 weeks old. Age-matched C57BL/6 mice (Jackson Laboratory) were used as controls with equivalent results, so all control results were pooled. All mice were housed in a specific pathogen-free barrier facility and screened regularly for pathogens. All studies and procedures were approved by the Committee on Animal Experimentation of Nagasaki University Graduate School of Biomedical Sciences and by the Committee on Animal Experimentation of Kanazawa University Graduate School of Medical Science.

Tolerance induction

Mice were painted 5 times (on days 0, 2, 4, 6, and 8) with tolerizing doses of 4.5 μg TNCB (Sigma-Aldrich, St. Louis, MO) in 15 μl of 3:1 acetone:olive oil onto shaved back skin.

CHS assay

On day 10, mice backs were shaved and painted with 450 μg TNCB in 15 μl 3:1 acetone:olive oil. On day15, mice were challenged on the dorsal surface of both ears with 45 μg of 15 μl 3:1 acetone:olive oil. Ear thickness was measured with a Peacock spring-loaded micrometer (Ozaki, Tokyo, Japan) before and 48 hours after challenge. The ear swelling reactions were expressed as the difference between the ear thickness

before and after challenge. Each ear lobe was measured three times at each time point, and the mean of those values was used for analysis.

Adoptive transfer of spleen cells

Donor mice were exposed 5 times (on days 0, 2, 4, 6, and 8) with tolerizing doses of TNCB on shaved backs, and sensitized with TNCB on their shaved backs on day 10. Seven days after sensitization, mice were killed, spleens harvested, and single-cell suspensions prepared. Viable spleen cells ($1x10^8$) were injected into the tail vein of naive recipients.

Enrichment CD8$^+$ and CD4$^+$ T cells for adoptive transfer and proliferative responses

CD8$^+$ and CD4$^+$ T cells were negatively depeleted from whole lymph node cells or splenocytes using CD8-corted (for CD4$^+$ enrichment) or CD4-corted (for CD8$^+$ enrichment) magnetic beads (Dynal).

Treatment with anti-IL-10 mAb

Immediately before antigen challenge, 40 μl of anti-IL-10 mAb (JES5.2A5, 1 mg/ml in PBS, BD-PharMingen, San Diego, CA) was injected intradermally near the ear using a 29-gauge needle under ether anesthesia. Injection of an equal volume of rat polyclonal IgG (1 mg/ml in PBS, Sigma) near the ears served as a control.

Histopathology

48 hours after antigen challenge, a central strip of the ear was collected, fixed in 3.5% paraformaldehyde, and then paraffin embedded. Six-micrometer sections were stained using H&E for general histological evaluation.

RNA Isolation and Real-Time Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from the ear using Pro Green kits (MP Biomedicals, Irvine, CA) in accordance with the manufacturer's protocol. Total RNA was reverse-transcribed to cDNA using a reverse transcription system with random hexamers (Promega, Madison, WI), and mRNA levels of ICAM-1 and TNF-α were analyzed by real-time RT-PCR using the TaqMan system (Applied Biosystems, Foster City, CA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to normalize mRNA. We obtained all sequence-specific primers and probes from TaqMan gene expression assays (Applied Biosystems). Real-time PCR was performed on an ABI Prism 7000 sequence detector or an ABI Prism 7300 sequence detector (Applied Biosystems) according to the manufacturer's instructions. Relative expression of real-time PCR products was determined using the C_T technique. In brief, we normalized each set of samples using the difference in threshold cycle (C_T) between the target gene and GAPDH: $C_T = (C_{T\ target\ gene} - C_{T\ GAPDH})$. Relative mRNA levels were calculated by the formula 2^{-CT} where $C_T = C_{T\ sample\ (n)} - C_{T\ calibrator\ (n)}$. Each reaction was done in triplicate. Samples from the same genotype without treatment were chosen as calibrator samples.

In vitro proliferative response to hapten

To assess in vitro proliferative responses to hapten, splenocytes or cervical and inguinal lymph nodes were collected 5 days after TNCB sensitization as previously described (Komura *et al.*, 2003). Single-cell suspensions (2×10^5 cells/well in 0.2 ml) were cultured in triplicate in 96-well plates in RPMI 1640 medium (Sigma) containing 10% FCS with up to 20 mM TNBS, a water soluble analog of TNCB (Sigma). Cellular proliferation was quantified by the addition of 10 μM 5-bromo-2'-deoxyuridine (BrdU; Roche Diagnostics, Mannheim, Germany) during

the last 18 hours of a 5-day culture, and BrdU incorporation was assayed by ELISA (Roche Diagnostics), according to the manufacturer's instructions.

Statistical analysis

The Mann-Whitney U test was used for determining the level of significance of differences. Bonferroni's test was used for multiple comparisons.

Acknowledgements

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Figure 1. Repeatedly painting tolerizing dose**s** of TNCB affects ICAM-1 mRNA production at CHS elicitation sites. mRNA was isolated from the ears 12 hours after challenge. ICAM-1 mRNA levels were analyzed by real-time RT-PCR and normalized with the GAPDH internal control. Each sample was done in triplicate. Data are the mean + SEM. These results represent those obtained with at least 10 mice in each treatment group.

Figure 2. Adhesion molecule deficiency impacts LZT. Mice were sensitized with TNCB on tolerized back skin following the ear challenge. Ear swelling responses were measured after 48 hours. Data are the mean + SD for 5 mice in each group. *P < 0.05 vs. CHS in wild type mice. **P < 0.01.

Figure 3. a. ICAM-1 deficient splenocytes proliferate in response to contact antigen. Mice were sensitized to TNCB. Five days later, spleens were isolated from mice with prior treatment, such as low dose tolerization (tolerized mice), sensitization (sensitized mice), and no treatment (CTL). Single-cell suspensions were cultured in vitro with varying amounts of TNBS for 5 days. b. ICAM-1 deficient lymphocytes proliferate in response to contact antigen. Mice were sensitized to TNCB. Five days later, cervical and inguinal lymphocyte were isolated from mice with prior treatment, such as low dose tolerization (tolerization), sensitization (sensitization). Single-cell suspensions with/without CD8 depletion (for CD4$^+$ cell enrichment) or CD4 depletion (for CD8$^+$ cell enrichment) were cultured in vitro with/without 20mM TNBS for 5 days. Proliferation was assessed by the incorporation of BrdU added during the last 18 hours of culture. Values represent mean OD ($\pm$ SEM) from triplicate cultures. Results are shown for one of three independent experiments with one mouse of each

genotype and treatment. *P < 0.05 vs. CTL in the same genotype. **P < 0.05 in the same genotype.

Figure 4. The role of ICAM-1 during the generation of LZT-induced suppressor cells. Naive recipients were adoptively transferred $1x10^8$ splenocytes with/without CD8 depletion from mice that had been sensitized to TNCB with low dose tolerization (tolerized), or without tolerization (sensitized). The recipients were subsequently sensitized with TNCB. Five days later, the mice were challenged on the ear and ear thickness was measured after 48 hours. Data are the mean + SD for 5 mice per group. *P < 0.001 vs. the same genotype receiving splenocytes from sensitized donors. Mice without adoptive transfer served as controls (CTL).

Figure 5. LZT affects the elicitation phase of the CHS response. The presence of suppressor cells was assessed by adoptive co-transfer of both effector and suppressor cells into naive recipients. Equal numbers ($1x10^8$) of tolerized and sensitized donor splenocytes were adoptively transferred. Recipients were challenged on the ear without sensitization and ear swelling was measured 48 hours later. Data are the mean + SD for 5 mice in each group. *P < 0.05 vs. sensitized donors without tolerization. Mice without adoptive transfer served as controls (CTL)

Figure 6. IL-10 blockade during the elicitation restores LZT. (**a**) TNF-α mRNA expression. mRNA was isolated from ears 12 hours after the challenge. mRNA levels were analyzed by real-time RT-PCR and normalized with the GAPDH internal control. Each sample was done in triplicate. Data are the mean + SEM. These results represent those obtained with at least 10 mice in each treatment group. (b) Effect of anti-IL-10 mAb injection at the elicitation site of LZT. Immediately before antigen challenge, either anti-IL-10 mAb (αIL-10 Ab) or rat polyclonal IgG (CTL Ab) was injected intradermally near the ear. Ear thickness was measured 48 hours after Ag

challenge. Data are the mean + SD for 5 mice in each group. (**c**) Effect of anti-IL-10 mAb treatment on TNF-α mRNA production. Mice were treated with either αIL-10 Ab or CTL Ab as described above and 12 hours after antigen challenge TNF-α mRNA expression was evaluated. These results were obtained with 5 mice per treatment group. Data are the mean + SEM. (**d**) Effect of anti-IL-10 mAb treatment on ICAM-1 production. Wild type mice were treated with either αIL-10 Ab or CTL Ab as described above and 12 hours after antigen challenge ICAM-1 mRNA was assessed. Data are the mean + SEM. *P < 0.05.   **P < 0.01.

Abbreviations: Ag, antigen; BrdU, 5-bromo-2'-deoxyuridine; CHS, contact hypersensitivity; C_T, threshold cycle; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; L-selectin$^{-/-}$, L-selectin-deficient; L-selectin/ICAM-1$^{-/-}$, lacking both ICAM-1 and L-selectin; LZT, low zone tolerance; TNBS, 2,4,6-trinitro-benzenesulfonic acid; TNCB, 2,4,6-trinitro-1chlorobenzene; TNF, tumor necrosis factor; and UV, ultraviolet.
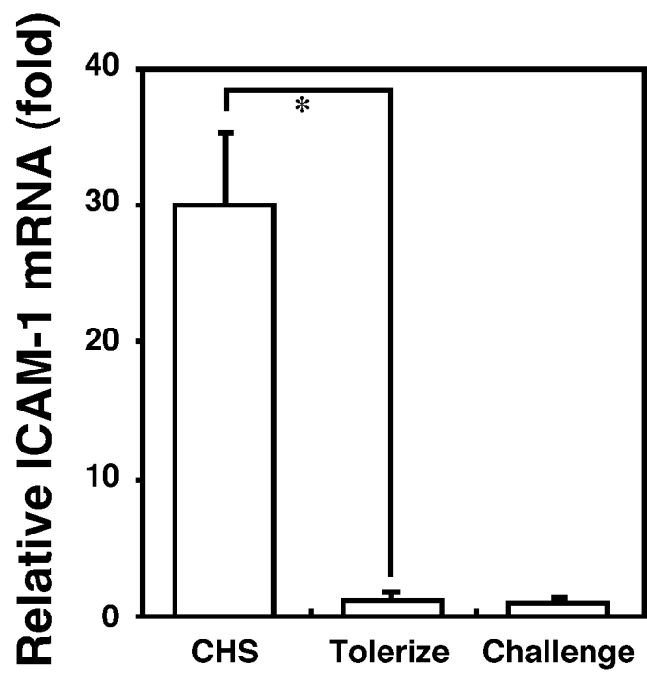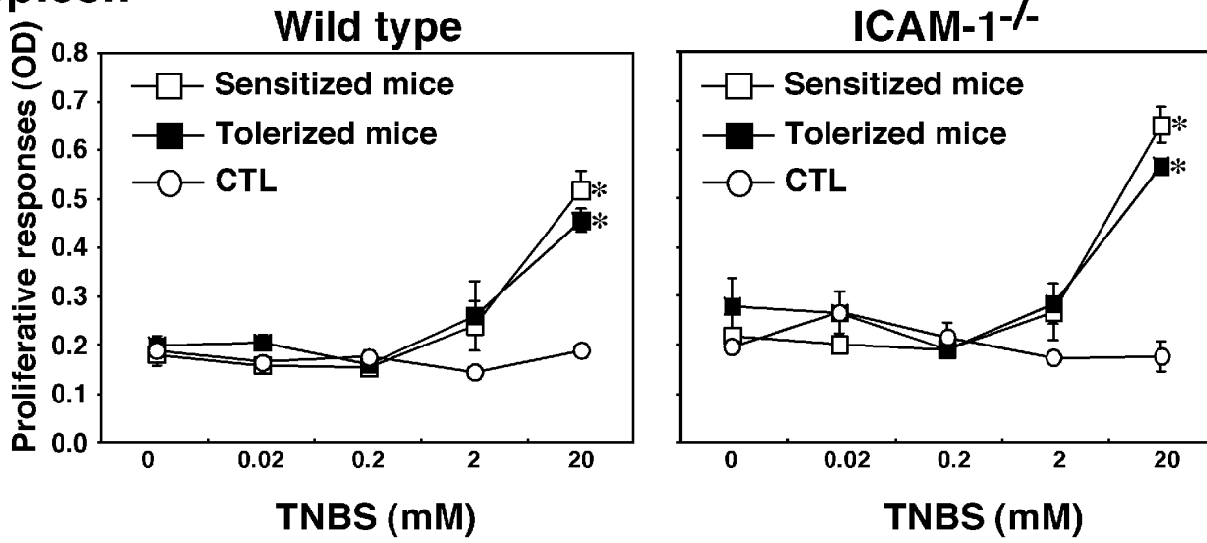
Figure 1
Komura, K et al.
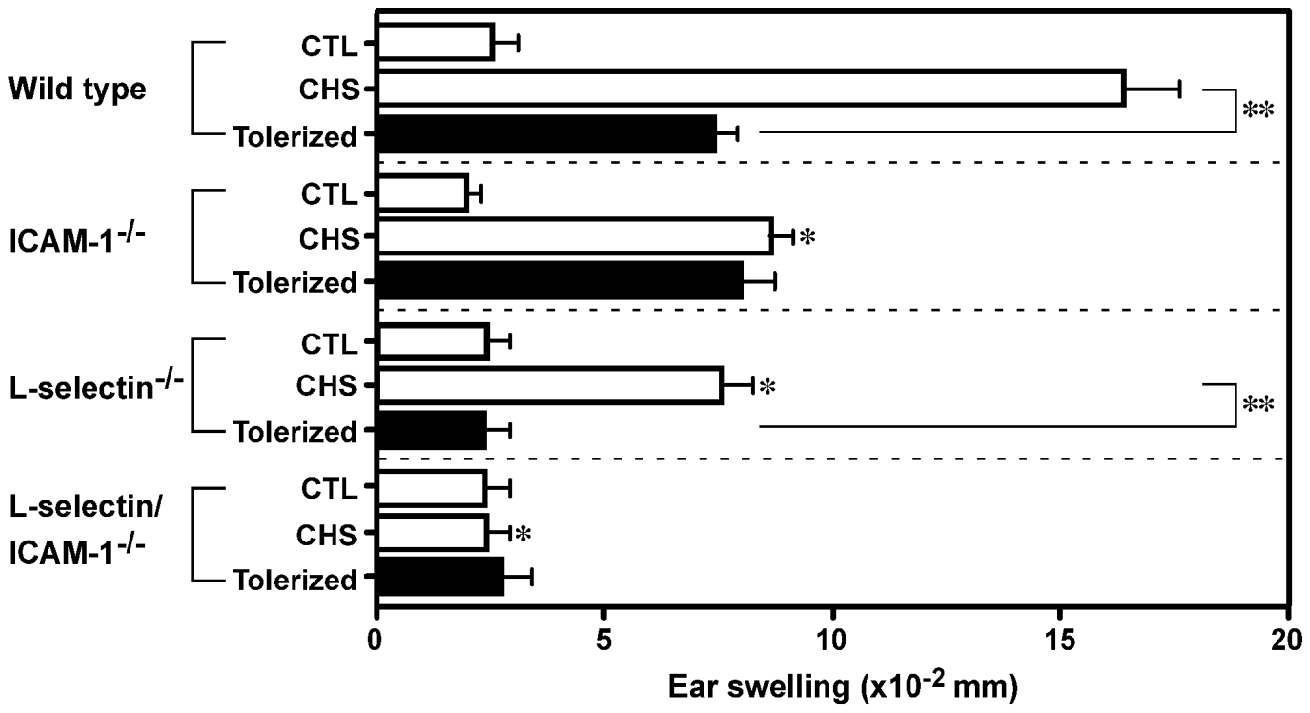
Figure 2
Komura, K et al.

a. Spleen

Wild type

ICAM-1⁻/⁻

b. Lymph Node

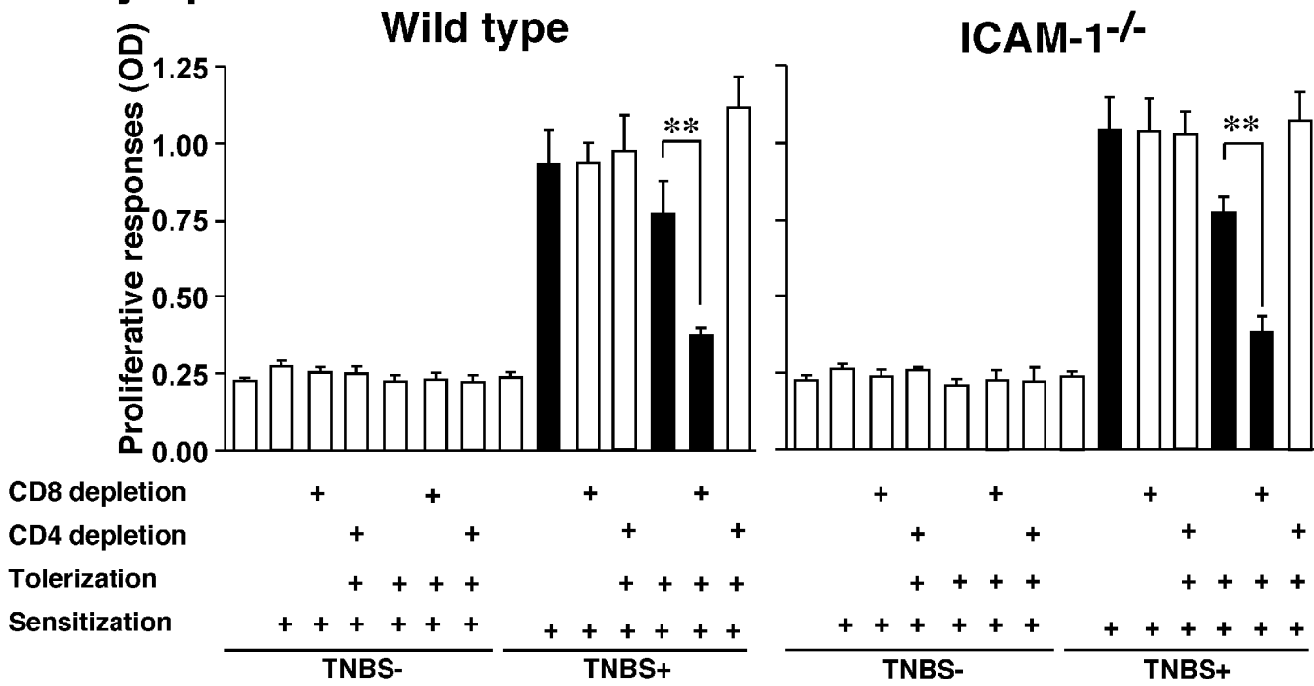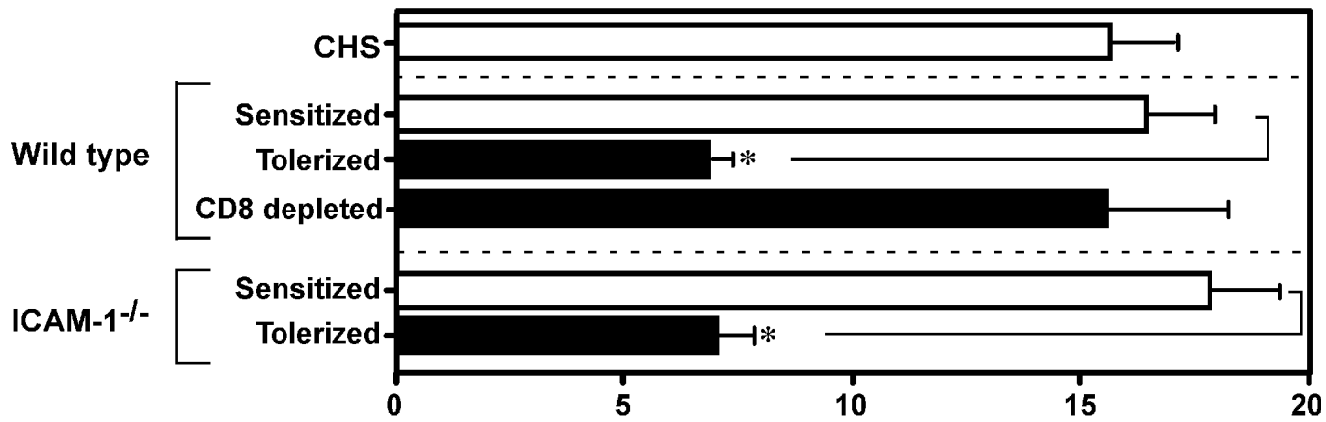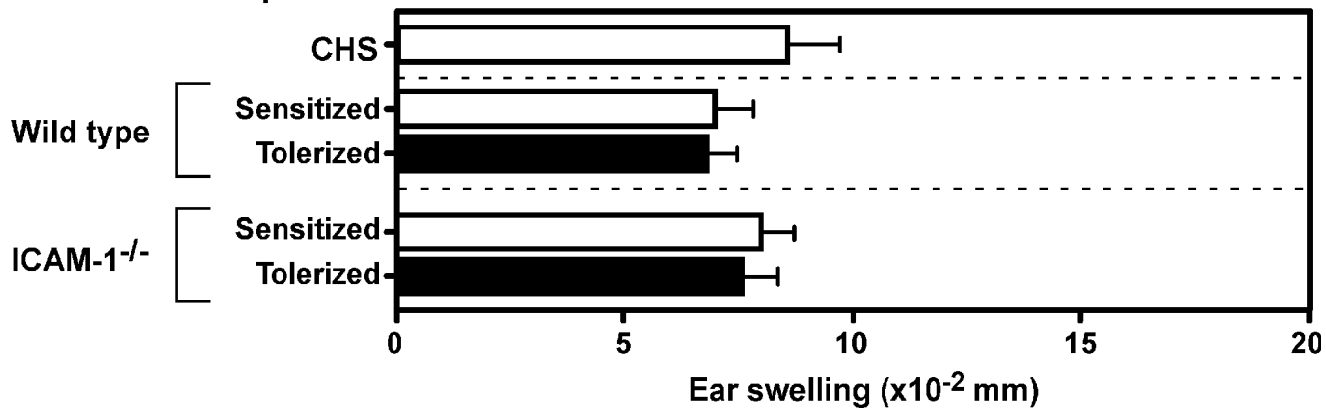## Wild type

ICAM-1⁻/⁻

Figure 3

Komura, K et al.
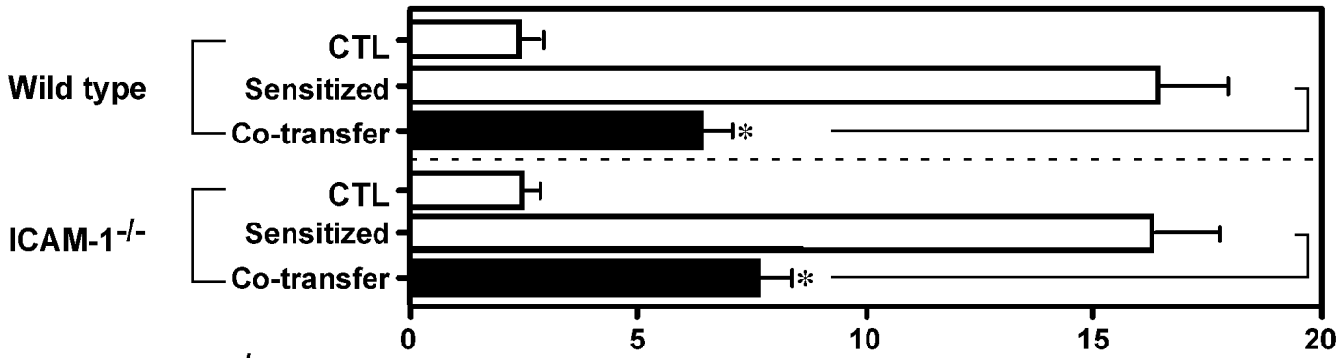
A. Wild type recipient

B. ICAM-1$^{-/-}$ recipient

Ear swelling (x10^{-2} mm)
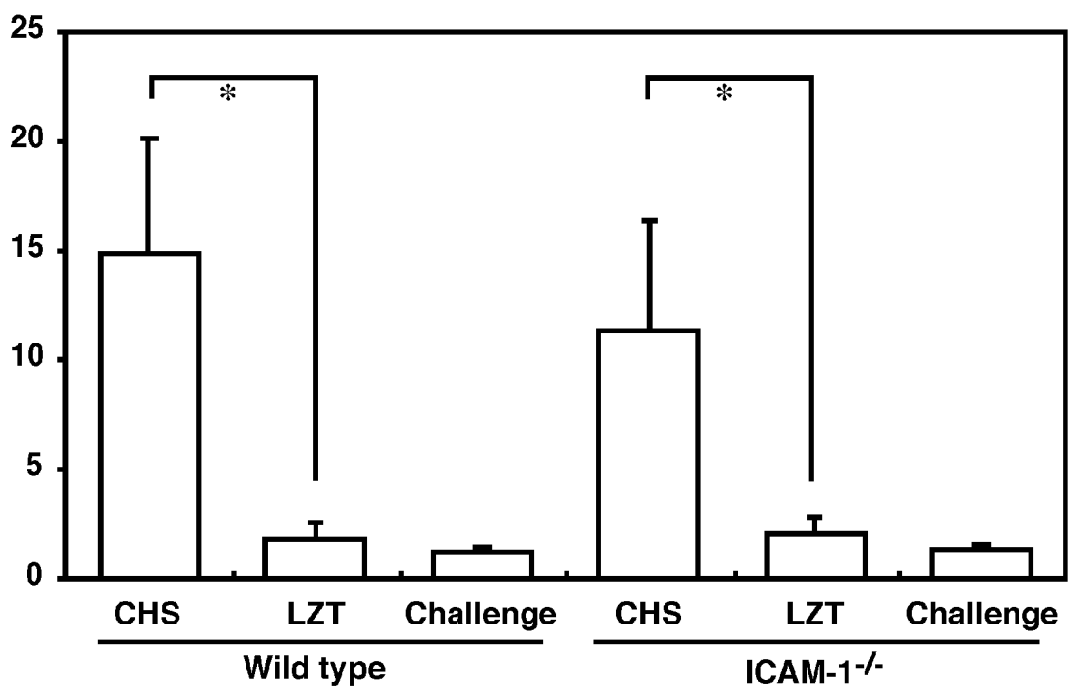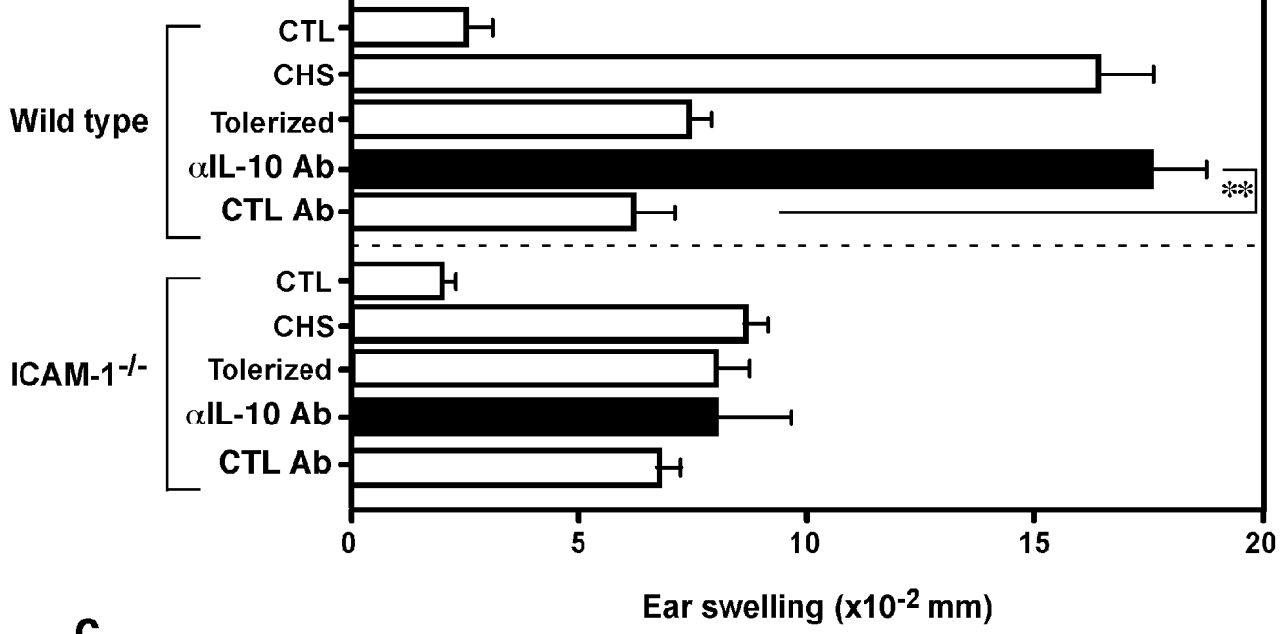
Figure 4
Komura, K et al.

A. Wild type recipient

B. ICAM-1⁻ᐟ⁻ recipient

Ear swelling (x10⁻² mm)
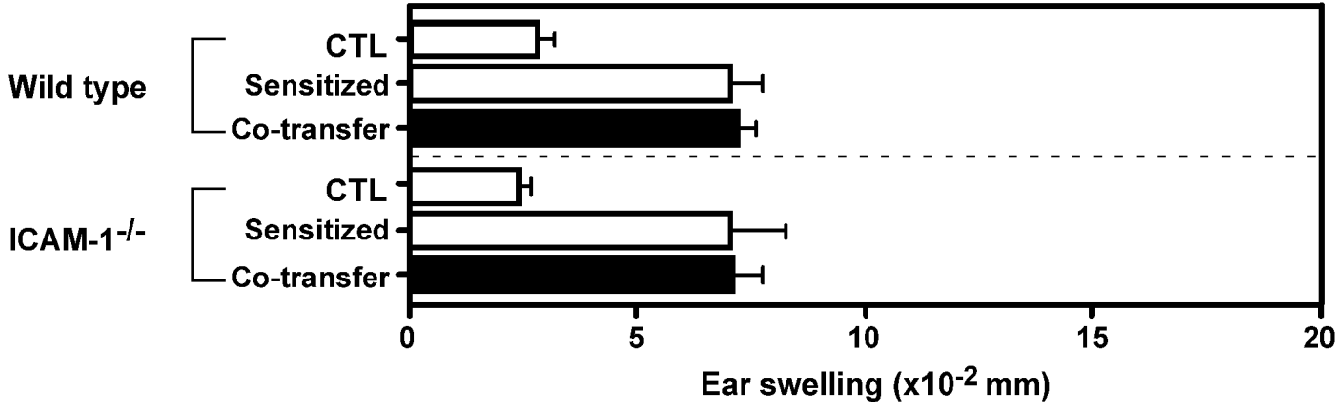
Figure 5
Komura, K et al.

a

Relative TNF-α mRNA (fold)

| CHS | LZT | Challenge | CHS | LZT | Challenge |

Wild type — ICAM-1⁻/⁻

b

Wild type:
CTL
CHS
Tolerized
αIL-10 Ab
CTL Ab

ICAM-1⁻/⁻:
CTL
CHS
Tolerized
αIL-10 Ab
CTL Ab

Ear swelling (x10⁻² mm)

0   5   10   15   20

c

TNF-α mRNA (fold)