Therapeutic Potential of B and T Lymphocyte Attenuator Expressed on CD8$^+$ T Cells for Contact Hypersensitivity

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In the past decade, mechanisms underlying allergic contact dermatitis have been intensively investigated by using contact hypersensitivity (CHS) models in mice. However, the regulatory mechanisms, which could be applicable for the treatment of allergic contact dermatitis, are still largely unknown. To determine the roles of B and T lymphocyte attenuator (BTLA), a CD28 family coinhibitory receptor, in hapten-induced CHS, BTLA-deficient (BTLA$^{-/-}$) mice and littermate wild-type (WT) mice were subjected to DNFB-induced CHS, severe combined immunodeficient (SCID) mice were injected with CD4$^+$ T cells, and CD8$^+$ T cells from either WT mice or BTLA$^{-/-}$ mice were subjected to CHS. BTLA$^{-/-}$ mice showed enhanced DNFB-induced CHS and proliferation and IFN-γ production of CD8$^+$ T cells as compared with WT mice. SCID mice injected with WT CD4$^+$ T cells and BTLA$^{-/-}$ CD8$^+$ T cells exhibited more severe CHS as compared with those injected with WT CD4$^+$ T cells and WT CD8$^+$ T cells. On the other hand, SCID mice injected with BTLA$^{-/-}$ CD4$^+$ T cells and WT CD8$^+$ T cells exhibited similar CHS to those injected with WT CD4$^+$ T cells and WT CD8$^+$ T cells. Finally, to evaluate the therapeutic potential of an agonistic agent for BTLA on CHS, the effects of an agonistic anti-BTLA antibody (6A6) on CHS were examined. *In vivo* injection of 6A6 suppressed DNFB-induced CHS and IFN-γ production of CD8$^+$ T cells. Taken together, these results suggest that stimulation of BTLA with agonistic agents has therapeutic potential in CHS.

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INTRODUCTION

Allergic contact dermatitis is a delayed type hypersensitivity reaction to foreign substances and hapten-modified proteins (Usatine and Riojas, 2010). Numerous studies using murine models of contact hypersensitivity (CHS), which is induced by epicutaneous exposure of haptens in sensitized mice, revealed detailed immunological mechanisms (Martin *et al.*, 2011; Kaplan *et al.*, 2012). During the sensitization phase of CHS, hapten-modified proteins are loaded onto epidermal Langerhans cells, which migrate from the epidermis to the regional draining lymph nodes, where priming of hapten-specific CD4$^+$ T cells and CD8$^+$ T cells occurs (Kaplan, 2010; Kaplan *et al.*, 2012). During the elicitation phase of CHS, hapten skin painting induces the recruitment of hapten-specific T cells into the skin, leading to the development of skin inflammation (Martin, 2004). Several studies have demonstrated that CHS to DNFB is regulated by the balance between IFN-γ-producing CD8$^+$ T cells as effector cells and IL-4-producing CD4$^+$ T cells as inhibitory cells (Vocanson *et al.*, 2009). However, regulatory mechanisms, which could be applicable for the treatment of allergic contact dermatitis, remain poorly understood.

The quality and quantity of T-cell responses are controlled not only by signals through T-cell receptors (TCRs) but also by the balance of secondary signals through costimulatory and coinhibitory receptors (Greenwald *et al.*, 2005). B and T lymphocyte attenuator (BTLA), a coinhibitory receptor with cytoplasmic immunoreceptor tyrosine-based inhibitory motifs (Murphy *et al.*, 2006; Cai and Freeman, 2009; Murphy and Murphy, 2010), has been reported to attenuate TCR-mediated signaling and proliferation of CD4$^+$ T cells and CD8$^+$ T cells (Murphy *et al.*, 2006; Cai and Freeman, 2009; Murphy and Murphy, 2010). *In vivo*, it has been shown that BTLA has inhibitory roles in a number of CD4$^+$ T-cell-mediated disease models (Watanabe *et al.*, 2003; Deppong *et al.*, 2006; Tamachi *et al.*, 2007). In addition, recent reports have

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Abbreviations: BTLA, B and T lymphocyte attenuator; CHS, contact hypersensitivity; DNBS, DNFB sulfonic acid; SCID, severe combined immunodeficient; TCR, T-cell receptor; WT, wild type

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revealed that BTLA also has a role in the regulation of $CD8^+$ T-cell function (Tao *et al.*, 2005; Krieg *et al.*, 2007; Liu *et al.*, 2009; Derré *et al.*, 2010). However, roles of BTLA in the regulation of hapten-induced CHS remain unknown.

In this study, we found that DNFB-induced CHS was significantly enhanced in $BTLA^{-/-}$ mice. Cell transfer experiments revealed that BTLA expressed on $CD8^+$ T cells had an inhibitory role in CHS. DNFB sulfonic acid (DNBS)–induced proliferation was enhanced in $BTLA^{-/-}$ $CD8^+$ T cells but not in $BTLA^{-/-}$ $CD4^+$ T cells. *In vivo* injection of an agonistic anti-BTLA antibody inhibited IFN-γ production of $CD8^+$ T cells and CHS. These results indicate that BTLA functions as a negative regulator of hapten-induced CHS by attenuating $CD8^+$ T-cell activation, and that BTLA could be a therapeutic target in allergic contact dermatitis.

RESULTS

DNFB-induced CHS is enhanced in $BTLA^{-/-}$ mice

To determine whether BTLA is involved in CHS, we first compared DNFB-induced CHS in $BTLA^{-/-}$ mice and littermate wild-type (WT) mice. $BTLA^{-/-}$ mice and WT mice in BALB/c background were sensitized on the shaved abdomen with DNFB and 5 days later, the ear was painted with DNFB. As shown in Figure 1, $BTLA^{-/-}$ mice exhibited enhanced ear thickness (Figure 1a) and severe inflammation (Figure 1b, $n=8$, $P<0.01$) as compared with WT mice. We next analyzed leukocytes recovered from the ear in mice challenged with DNFB by flow cytometry and found that the numbers of $CD4^+$ T cells, $CD8^+$ T cells, Gr-1^+ cells, and F4/80^+ cells were increased in $BTLA^{-/-}$ mice as compared with those in WT mice (Figure 1c, $n=6$, $P<0.01$). These results indicate that BTLA functions as an attenuator in the development of hapten-induced CHS.

Importantly, $CD8^+$ T cells expressed lower levels of BTLA than $CD4^+$ T cells at the inflammatory sites (Figure 1d; $CD4^+$ 76.8 ± 5.8 vs. $CD8^+$ 18.3 ± 3.5; mean fluorescence intensity, $n=5$, $P<0.01$), whereas the expression levels of BTLA on naive $CD8^+$ T cells in lymph nodes were similar to those on naive $CD4^+$ T cells (Supplementary Figure S1 online). On the other hand, DNFB challenge did not change the expression of herpesvirus entry mediator, a ligand of BTLA, on dermal dendritic cells and Langerhans cells (Supplementary Figure S2 online). Moreover, $CD8^+$ T cells recovered from the ear in DNFB-challenged $BTLA^{-/-}$ mice produced higher levels of IFN-γ than those in DNFB-challenged WT mice (WT 218.4 ± 8.8 vs. $BTLA^{-/-}$ 332.5 ± 10.5; mean fluorescence intensity, $n=5$, $P<0.01$) (Figure 1e), suggesting that BTLA limits $CD8^+$ T-cell-mediated functions on a per cell basis. Taken together with previous studies showing that the expression of BTLA on $CD8^+$ T cells is downregulated in effector $CD8^+$ T cells (Derré *et al.*, 2010; Serriari *et al.*, 2010), these results suggest that $CD8^+$ T cells at the site of CHS in $BTLA^{-/-}$ mice are in an effector state.

Effector $CD8^+$ T cells are increased in the draining lymph nodes in DNFB-sensitized $BTLA^{-/-}$ mice

To address the mechanisms underlying the enhanced DNFB-induced CHS in $BTLA^{-/-}$ mice, we next examined the activation status of T cells in the draining lymph nodes in DNFB-sensitized $BTLA^{-/-}$ mice and WT mice. Although no significant difference was found in the frequency of effector $CD4^+$ T cells ($CD44^{high}$ $LY6C^{low}$ $CD4^+$ cells) in $BTLA^{-/-}$ mice and WT mice (Figure 2a, $n=5$), the frequency of effector $CD8^+$ T cells ($CD44^{high}$ $LY6C^{low}$ $CD8^+$ cells) was significantly increased in the draining lymph nodes of DNFB-sensitized $BTLA^{-/-}$ mice as compared with those of DNFB-sensitized WT mice (Figure 2b, $n=5$, $P<0.01$). On the other hand, the expression levels of CXCR3, a chemokine receptor allowing effector T cells to enter peripheral tissues (Zhang and Bevan, 2011), on $CD44^{high}$ $LY6C^{low}$ $CD8^+$ cells were similar in DNFB-sensitized $BTLA^{-/-}$ mice and WT mice (Figure 2c, $n=5$). These data suggest that in addition to the inhibitory effect of BTLA on $CD8^+$ T-cell-mediated functions on a per cell basis (Figure 1e), the enhanced expansion of effector $CD8^+$ T cells could be involved in the enhanced DNFB-induced CHS in $BTLA^{-/-}$ mice.

It has been shown that regulatory T cells inhibit CHS by attenuating the expansion of hapten-stimulated $CD8^+$ T cells (Dubois *et al.*, 2003; Ring *et al.*, 2006; Cavani. 2008; Vocanson *et al.*, 2009). Therefore, we next examined $FOXP3^+$ $CD4^+$ T cells in the draining lymph nodes of DNFB-sensitized $BTLA^{-/-}$ mice and WT mice. As shown in Supplementary Figure S3a online, the frequency of $FOXP3^+$ $CD4^+$ T cells in the draining lymph nodes of DNFB-sensitized $BTLA^{-/-}$ mice was similar to those in DNFB-sensitized WT mice ($n=5$). The expression levels of GITR, CTLA4, and CD25 on $FOXP3^+$ $CD4^+$ T cells were also similar in DNFB-sensitized $BTLA^{-/-}$ mice and WT mice (Supplementary Figure S3b online), indicating that regulatory T cells are similarly developed during CHS in $BTLA^{-/-}$ mice and WT mice. Recent studies have shown that among regulatory T cells, an $ICOS^+$ subpopulation strongly inhibits hapten-specific $CD8^+$ T cells as compared with an $ICOS^-$ subpopulation (Vocanson *et al.*, 2010; Gomez de Agüero *et al.*, 2012). However, as shown in Supplementary Figure S4 online, the frequency of $ICOS^+$ $CD25^+$ T cells in the draining lymph nodes of DNFB-sensitized $BTLA^{-/-}$ mice was similar to that of DNFB-sensitized WT mice ($n=5$). We also found that the number of $FOXP3^+$ $CD4^+$ T cells recovered from the ear and IL-10 production in $FOXP3^+$ $CD4^+$ T cells in DNFB-challenged $BTLA^{-/-}$ mice were similar to those in DNFB-challenged WT mice (Supplementary Figure S5 online). These results suggest that BTLA expressed on $FOXP3^+$ $CD4^+$ T cells may not be involved in the regulation of hapten-induced CHS.

BTLA expressed on $CD8^+$ T cells is involved in the attenuation of DNFB-induced CHS

To address the cellular mechanisms underlying the enhanced DNFB-induced CHS in $BTLA^{-/-}$ mice, SCID mice that were injected with a mixture of $CD4^+$ T cells and $CD8^+$ T cells from either $BTLA^{-/-}$ mice or WT mice were subjected to DNFB-induced CHS. Importantly, SCID mice injected with WT $CD4^+$ T cells and $BTLA^{-/-}$ $CD8^+$ T cells exhibited enhanced ear thickness ($n=8$, $P<0.01$) (Figure 3a), severe inflammation in the ear ($n=8$) (Figure 3b), and increased numbers of IFN-γ-producing $CD8^+$ T cells in the ear ($n=6$,

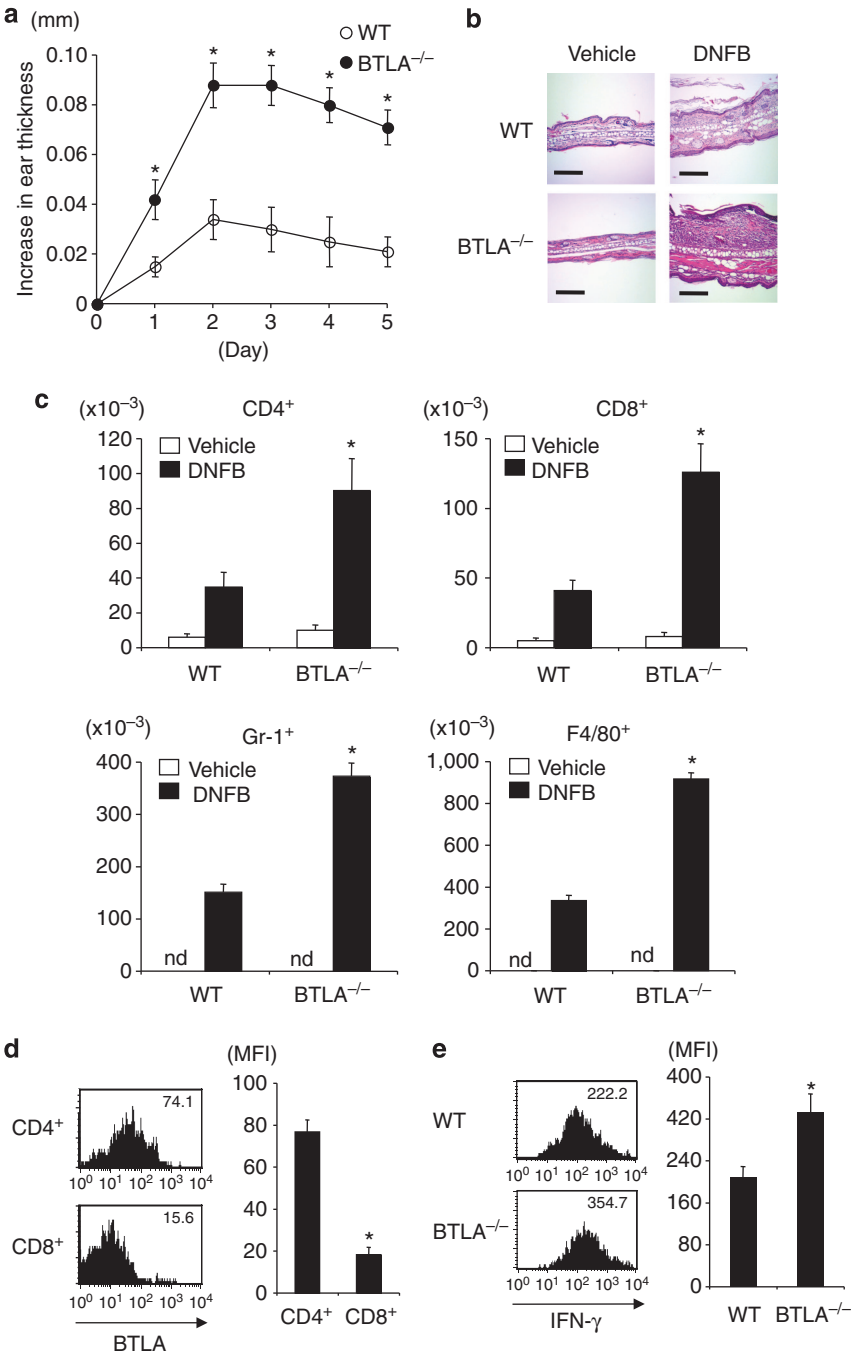

Figure 1. Contact hypersensitivity is enhanced in B and T lymphocyte attenuator–deficient (BTLA$^{-/-}$) mice. BTLA$^{-/-}$ mice and littermate wild-type (WT) mice on the BALB/c background were sensitized with DNFB, and 5 days after DNFB sensitization, mice were challenged with DNFB on the ear. (**a**) Ear swelling was quantified by the measurements of ear thickness before (baseline) and 1, 2, 3, 4, and 5 days after the challenge. Shown are means ± SD ($n = 8$ mice in each group) of the increase in the ear thickness from the baseline. *Significantly different from the mean value of WT mice, *$P < 0.01$. (**b**) Five days after the challenge with DNFB, specimens of the ear from BTLA$^{-/-}$ mice ($n = 6$) and littermate WT mice ($n = 6$) were stained with hematoxylin and eosin. Representative photomicrographs are shown. (**c**) Five days after the challenge with DNFB, leukocytes were isolated from the ear and analyzed as described in the Materials and Methods. Shown are means ± SD ($n = 6$ mice in each group) of the numbers of CD4$^+$ cells, CD8$^+$ cells, Gr-1$^+$ cells, and F4/80$^+$ cells. *Significantly different from the mean value of WT mice, *$P < 0.01$. (**d**) Five days after the challenge with DNFB, the expression of BTLA on CD4$^+$ cells and CD8$^+$ cells isolated from the ear was examined by flow cytometry. Shown are representative histograms with mean fluorescence intensity (MFI) and means ± SD ($n = 5$, each) of the MFI of anti-BTLA staining. *$P < 0.01$. (**e**) Two days after the challenge with DNFB, the expression of IFN-γ in CD8$^+$ cells isolated from the ear was examined by flow cytometry. Shown are representative histograms with MFI and means ± SD ($n = 5$, each) of the MFI of anti-IFN-γ staining. *$P < 0.01$. Data are representative of three independent experiments. nd, not detectable. Bar = 200 μm.

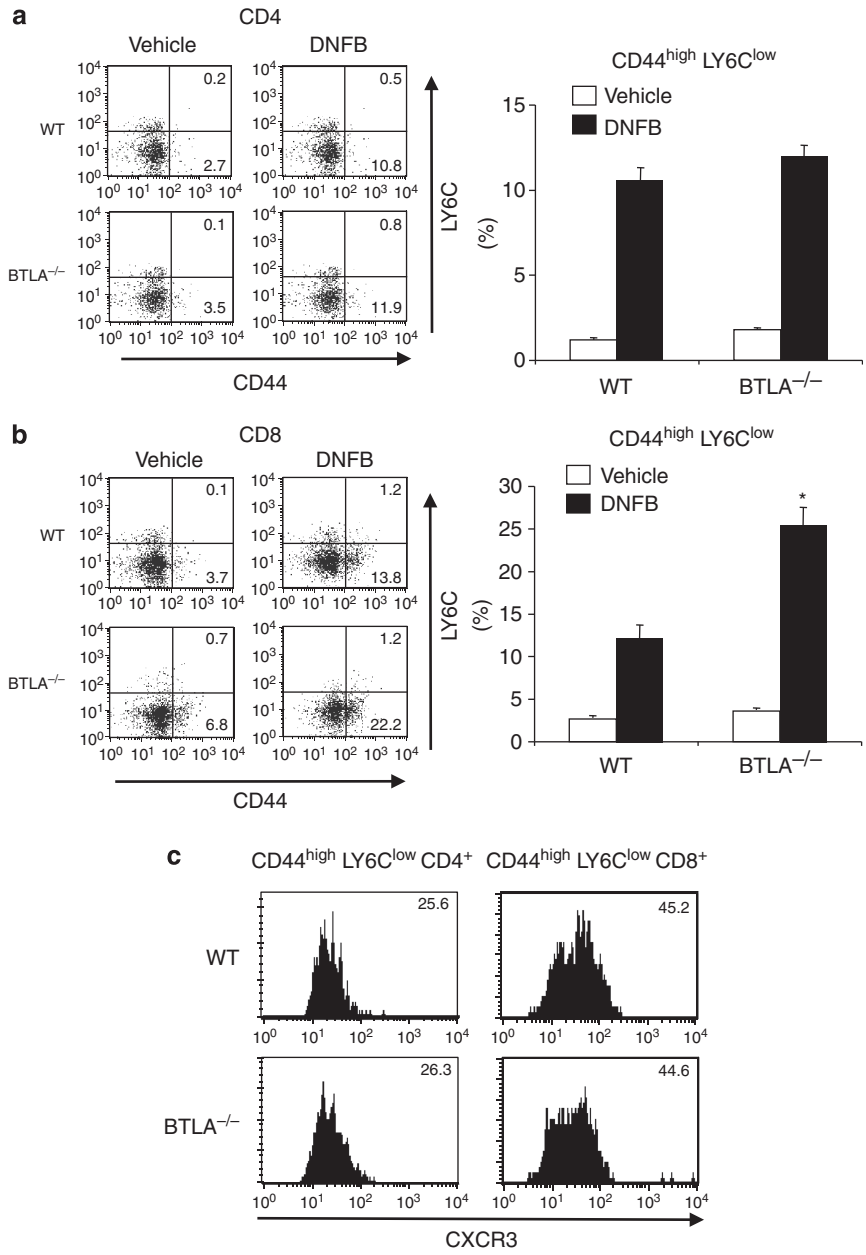

Figure 2. Effector CD8$^+$ T cells but not CD4$^+$ T cells are increased in the draining lymph nodes of DNFB-sensitized B and T lymphocyte attenuator–deficient (BTLA$^{-/-}$) mice. BTLA$^{-/-}$ mice and littermate wild-type (WT) mice were sensitized with DNFB on the arms and legs. Five days after the sensitization, cells from inguinal and axillary lymph nodes were analyzed by flow cytometry. (**a**, **b**) Shown are representative dot plots of CD44 vs. LY6C staining and means ± SD ($n=5$, each) of the frequency of effector population (CD44^{high} LY6C^{low} cells) of (**a**) CD4$^+$ T cells and (**b**) CD8$^+$ T cells. *$P<0.01$. (**c**) Shown are representative histograms for the CXCR3 expression on CD44^{high} LY6C^{low} CD4$^+$ cells or CD44^{high} LY6C^{low} CD8$^+$ cells. $n=5$. Data are representative of three independent experiments.

$P<0.01$) (Figure 3c) as compared with those injected with WT CD4$^+$ T cells and WT CD8$^+$ T cells. Similarly, SCID mice injected with BTLA$^{-/-}$ CD4$^+$ T cells and BTLA$^{-/-}$ CD8$^+$ T cells exhibited enhanced ear thickness, severe inflammation in the ear, and increased numbers of IFN-γ-producing CD8$^+$ T cells in the ear as compared with those injected with BTLA$^{-/-}$ CD4$^+$ T cells and WT CD8$^+$ T cells (Figure 3, $P<0.01$). These results suggest that BTLA expressed on CD8$^+$ T cells is involved in the attenuation of DNFB-induced CHS.

On the other hand, SCID mice injected with BTLA$^{-/-}$ CD4$^+$ T cells and WT CD8$^+$ T cells exhibited similar DNFB-induced CHS to those injected with WT CD4$^+$ T cells and WT CD8$^+$ T cells, suggesting that BTLA expressed on CD4$^+$ T cells is not significantly involved in the regulation of DNFB-induced CHS.

DNBS-induced cell proliferation, cytokine production, and exocytosis are enhanced in BTLA$^{-/-}$ CD8$^+$ T cells

To clarify the basis for the enhanced CHS in BTLA$^{-/-}$ mice in detail, hapten-induced proliferation and cytokine production of CD4$^+$ T cells and CD8$^+$ T cells in BTLA$^{-/-}$ mice were

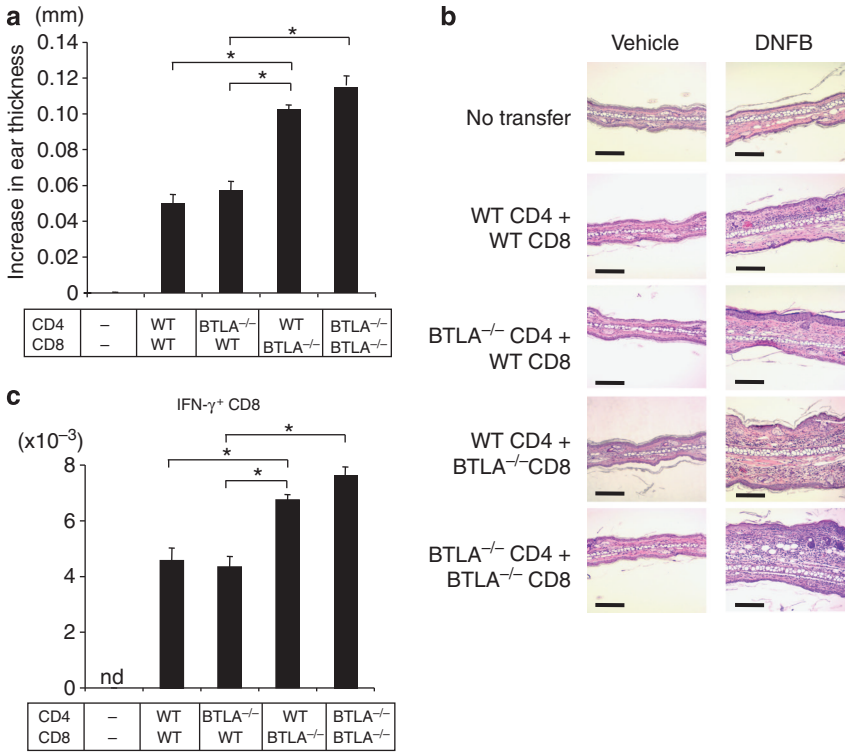

Figure 3. B and T lymphocyte attenuator (BTLA) expressed on CD8$^+$ T cells is involved in the attenuation of contact hypersensitivity (CHS). CD4$^+$ T cells and CD8$^+$ T cells were isolated from the spleen of BTLA$^{-/-}$ mice and wild-type (WT) mice, and a mixture of CD4$^+$ T cells (1×10^6 cells) and CD8$^+$ T cells (1×10^6 cells) from either BTLA$^{-/-}$ mice or WT mice were transferred intravenously to severe combined immunodeficient mice. The mice were sensitized and challenged with DNFB to elicit contact hypersensitivity as described in Materials and Methods. (**a**) Shown are means ± SD ($n = 8$ mice in each group) of the increase in the ear thickness from the baseline at 2 days after DNFB challenge. *$P<0.01$. (**b**) Five days after the challenge, specimens of the ear were stained with hematoxylin and eosin. Representative photomicrographs are shown. $n = 8$ mice in each group. (**c**) Five days after the challenge, leukocytes were isolated from the ear and the number of IFN-γ-producing CD8$^+$ T cells were evaluated as described in the Materials and Methods. Shown are means ± SD ($n = 6$ mice in each group) of the numbers of IFN-γ$^+$ CD8$^+$ cells recovered from the ear. *$P<0.01$. Data are representative of two independent experiments. nd, not detectable. Bar = 200 μm.

compared with those in WT mice. Five days after the sensitization with DNFB, CD62L^{low} CD4$^+$ T cells and CD62L^{low} CD8$^+$ T cells were isolated from the draining lymph nodes in WT mice and BTLA$^{-/-}$ mice, and stimulated with DNBS in the presence of irradiated splenic antigen-presenting cells. Importantly, BTLA$^{-/-}$ CD8$^+$ T cells showed enhanced DNBS-induced proliferation (Figure 4a, $n = 6$, $P<0.01$) and IFN-γ production (Figure 4b, $n = 6$, $P<0.01$) as compared with WT CD8$^+$ T cells, whereas BTLA$^{-/-}$ CD4$^+$ T cells showed similar levels of DNBS-induced proliferation (Figure 4a) and IL-4 production (Figure 4b) to WT CD4$^+$ T cells. As the release of perforin from CD8$^+$ T cells by exocytosis has been shown to be involved in CHS (Kehren *et al.*, 1999), we investigated DNBS-induced exocytosis of CD8$^+$ T cells by evaluating the surface expression of CD107a, a marker of exocytosis of lymphocytes (Betts *et al.*, 2003). The expression of CD107a was significantly increased in BTLA$^{-/-}$ CD8$^+$ T cells on DNBS stimulation as compared with WT CD8$^+$ T cells (WT 60.2 ± 5.7 vs. BTLA$^{-/-}$ 101.4 ± 5.5; mean fluorescence intensity, $n = 5$, $P<0.01$) (Figure 4c). These results indicate that BTLA attenuates not only hapten-induced proliferation of CD8$^+$ T cells but also their functions on a per cell basis, suggesting that this attenuation could be involved in the downregulation of hapten-induced CHS.

An agonistic anti-BTLA mAb prevents CHS

Finally, to evaluate the therapeutic potential of BTLA stimulation for allergic contact dermatitis, we examined the effect of an agonistic anti-BTLA mAb (6A6) on DNFB-induced CHS in WT mice (C57BL/6 background). As shown in Figure 5a, 6A6 significantly suppressed ear thickness ($n = 6$, $P<0.001$). Importantly, even if 6A6 was injected to mice at 24 hours after DNFB challenge, when CHS was being established, 6A6 suppressed ear thickness (Figure 5a). The injection of 6A6 at 24 hours after DNFB challenge also decreased the numbers of CD4$^+$ T cells, CD8$^+$ T cells, Gr-1$^+$ cells, and F4/80$^+$ cells recovered from the ear (Figure 5b, $n = 6$, $P<0.01$). As expected, the injection of 6A6 did not attenuate DNFB-induced CHS in BTLA$^{-/-}$ mice (Supplementary Figure S6 online), confirming that the inhibitory effect of 6A6 on CHS is mediated by BTLA. The injection of 6A6 at 24 hours after DNFB challenge also significantly decreased the number of IFN-γ-producing CD8$^+$ T cells in the ear (Figure 5c, $n = 6$, $P<0.01$). On the other hand, the injection of 6A6 did not reduce the numbers of CD4$^+$ T cells, CD8$^+$ T cells, and

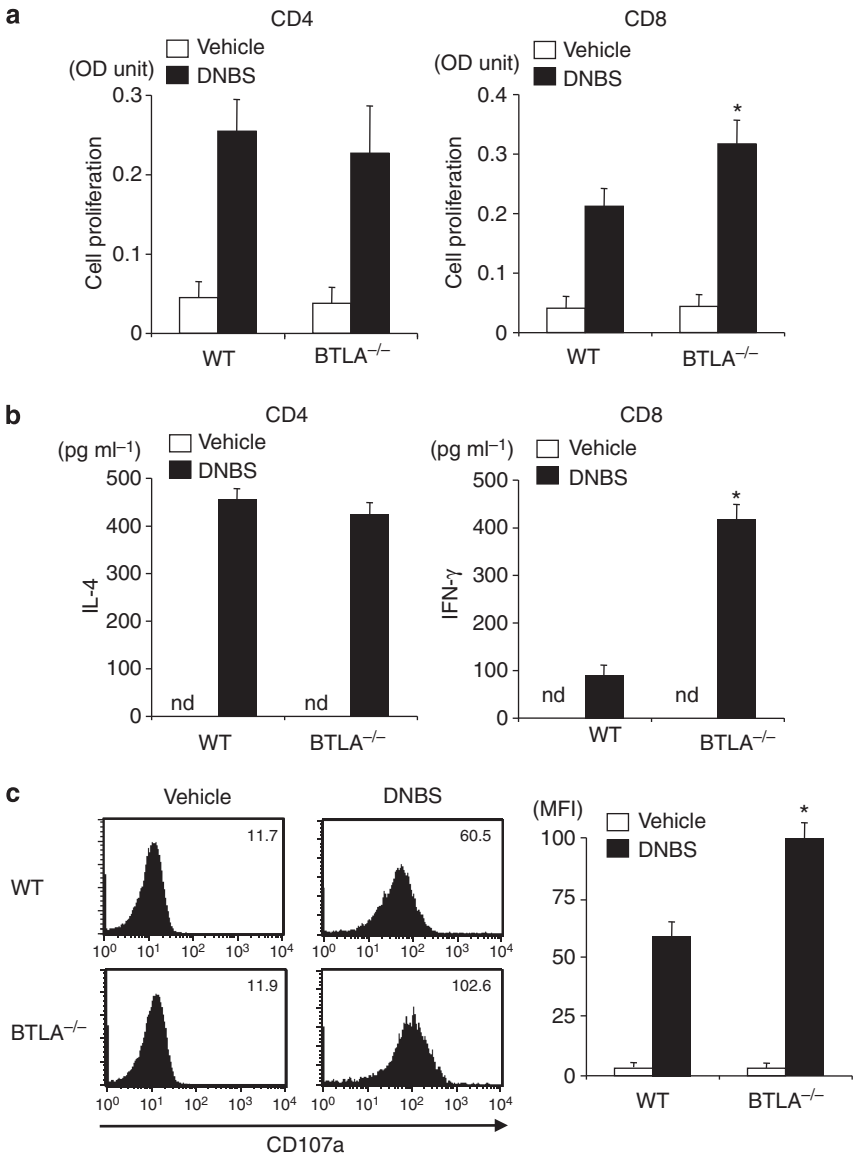

Figure 4. 2,4-Dinitrofluorobenzene sulfonic acid (DNBS)–induced cell proliferation, cytokine production, and exocytosis are enhanced in B and T lymphocyte attenuator–deficient (BTLA$^{-/-}$) CD8^{+} T cells. BTLA$^{-/-}$ mice and littermate wild-type (WT) mice were sensitized with DNFB on the arms and legs and 5 days after the sensitization, CD62L^{low} CD4^{+} T cells and CD62L^{low} CD8^{+} T cells were isolated from inguinal and axillary lymph nodes. Cells were then stimulated with DNBS or vehicle (as a control) in the presence of antigen-presenting cells, and (**a**) cell proliferation, (**b**) IL-4 production from CD62L^{low} CD4^{+} T cells and IFN-γ production from CD62L^{low} CD8^{+} T cells, and (**c**) CD107a expression on CD62L^{low} CD8^{+} T cells were evaluated. Data are (**a**) means ± SD of optical density (OD) unit at 450 nm ($n=6$), (**b**) the levels of IL-4 and IFN-γ in the supernatants ($n=6$), (**c**) and the representative histograms and means ± SD of the MFI of CD107a expression ($n=5$). *$P<0.01$. Data are representative of three independent experiments. nd, not detectable.

FOXP3^{+}CD4^{+} T cells in the spleen of WT mice (Supplementary Figure S7 online). We also confirmed the binding of 6A6 on CD8^{+} T cells in the ear of DNFB-challenged WT mice (Figure 5d). Taken together, these results suggest that 6A6 suppresses CHS by transducing inhibitory signal through BTLA in CD8^{+} T cells rather than by depleting cells expressing BTLA.

DISCUSSION

In this study, we show that BTLA expressed on CD8^{+} T cells attenuates hapten-induced CHS presumably through the suppression of proliferation and IFN-γ production of effector CD8^{+} T cells, and that agonistic agents for BTLA could be a new therapeutic approach for CHS. We found that DNFB-induced CHS was significantly enhanced in BTLA$^{-/-}$ mice (Figure 1). We also found that effector CD8^{+} T cells were increased in the draining lymph nodes in DNFB-sensitized BTLA$^{-/-}$ mice (Figure 2), and that SCID mice injected with BTLA$^{-/-}$ CD8^{+} T cells exhibited enhanced CHS as compared with those injected with WT CD8^{+} T cells (Figure 3). Furthermore, DNBS-induced proliferation, IFN-γ production, and exocytosis were enhanced in BTLA$^{-/-}$ CD8^{+} T cells

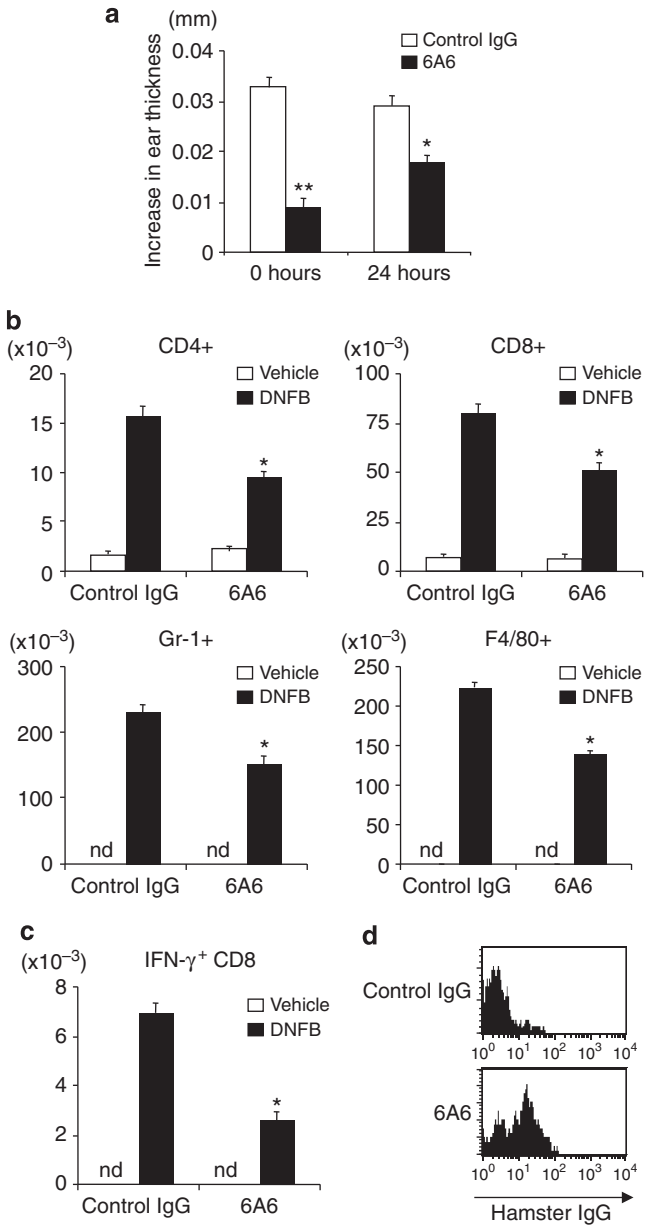

Figure 5. *In vivo* injection of an agonistic anti-B and T lymphocyte attenuator (anti-BTLA) mAb inhibits contact hypersensitivity. C57BL/6 mice were sensitized with DNFB and 5 days later mice were challenged with DNFB on the ear. Either just before or 24 hours after DNFB challenge, an agonistic anti-BTLA mAb (6A6) or control IgG was injected intraperitoneally to the mice. (**a**) Ear swelling was quantified at 2 days after the challenge. Shown are means ± SD ($n=6$ mice in each group) of the increase in the ear thickness from the baseline. *Significantly different from the mean value of mice injected with control IgG, **$P<0.001$, *$P<0.01$. (**b**) Two days after the challenge, leukocytes were isolated from the ear and analyzed by flow cytometry. Shown are means ± SD ($n=6$ mice in each group) of the numbers of $CD4^+$ cells, $CD8^+$ cells, Gr-1$^+$ cells, and F4/80$^+$ cells. *$P<0.01$. Two days after the challenge, leukocytes were isolated from the ear and (**c**) the number of IFN-γ-producing $CD8^+$ T cells and (**d**) the binding of hamster IgG (6A6 or control IgG) on $CD8^+$ cells were analyzed by flow cytometry. Shown are means ± SD of (**c**) the numbers of IFN-γ$^+$ $CD8^+$ cells recovered from the ear ($n=6$ mice in each group, *$P<0.01$) and (**d**) representative histograms for hamster IgG binding on $CD8^+$ cells ($n=5$ mice in each group). Data are representative of three independent experiments. nd, not detectable.

(Figure 4). Finally, we showed that *in vivo* administration of an agonistic anti-BTLA mAb suppressed CHS (Figure 5). Taken together, these results suggest that BTLA expressed on $CD8^+$ T cells could be a therapeutic target for allergic contact dermatitis.

We show that BTLA is involved in the attenuation of hapten-induced CHS. Coinhibitory molecules of CD28 family, including CTLA4, programmed cell death 1 (PD-1), and BTLA, have been shown to attenuate T-cell-mediated immune responses to varying degrees in various experimental settings (Chen, 2004). Previous studies have demonstrated that CTLA4 (Nuriya *et al.*, 2001) and PD-1 (Tsushima *et al.*, 2003) are involved in the attenuation of hapten-induced CHS by inhibiting the expansion of hapten-specific T cells. In this study, we found that BTLA$^{-/-}$ mice exhibited enhanced hapten-induced CHS (Figure 1) and the expansion of effector $CD8^+$ T cells (Figure 2). Taken together, it is indicated that all of the coinhibitory molecules of the CD28 family participate in the attenuation of hapten-induced CHS.

We also show that BTLA expressed on $CD8^+$ T cells is responsible for the attenuation of hapten-induced CHS. Previous studies investigating the roles of CTLA4 (Nuriya *et al.*, 2001) and PD-1 (Tsushima *et al.*, 2003) in hapten-induced CHS have not identified cell types whose expression of these coinhibitory molecules are responsible for the attenuation of CHS. On the other hand, we showed that SCID mice injected with WT $CD4^+$ T cells and BTLA$^{-/-}$ $CD8^+$ T cells exhibited enhanced CHS as compared with those injected with WT $CD4^+$ T cells and WT $CD8^+$ T cells (Figure 3), whereas hapten-induced CHS was similarly induced in SCID mice injected with BTLA$^{-/-}$ $CD4^+$ T cells and WT $CD8^+$ T cells and those injected with WT $CD4^+$ T cells and WT $CD8^+$ T cells (Figure 3). We also found that hapten-induced proliferation, IFN-γ production, and exocytosis were enhanced in BTLA$^{-/-}$ $CD8^+$ T cells (Figure 4), whereas hapten-induced proliferation and IL-4 production were indistinguishable between WT $CD4^+$ T cells and BTLA$^{-/-}$ $CD4^+$ T cells (Figure 4). Given that IFN-γ production (Vocanson *et al.*, 2009) and perforin release by exocytosis (Kehren *et al.*, 1999) are key events in $CD8^+$ T-cell-mediated induction of CHS, our results suggest that BTLA expressed on $CD8^+$ T cells attenuates hapten-induced CHS through the inhibition of hapten-induced IFN-γ production and perforin release of $CD8^+$ T cells.

Our results indicate that BTLA has inhibitory roles in CHS in both the sensitization and the elicitation phase. We found that effector $CD8^+$ T cells were increased in the draining lymph nodes in DNFB-sensitized BTLA$^{-/-}$ mice (Figure 2). We also found that DNBS-induced proliferation, IFN-γ production, and exocytosis were enhanced in BTLA$^{-/-}$ $CD8^+$ T cells recovered from draining lymph nodes (Figure 4), indicating that BTLA is involved in the sensitization phase of CHS. In addition, we showed that *in vivo* injection of an agonistic anti-BTLA mAb at 24 hours after DNFB challenge inhibited CHS (Figure 5a), indicating that BTLA is involved in the attenuation of CHS in the elicitation phase.

We found that not only the number of $CD8^+$ T cells but also the numbers of $CD4^+$ T cells, Gr-1$^+$ cells, and F4/80$^+$

cells were increased at the site of CHS in BTLA$^{-/-}$ mice as compared with those of WT mice (Figure 1c). We also found that the injection of the agonistic anti-BTLA antibody 6A6 at 24 hours after DNFB challenge suppressed the recruitment of various cell populations into ear skin (Figure 5b). In this regard, a recent study has shown that CD8^{+} T-cell-derived IFN-γ promotes the expression of proinflammatory cytokines and chemokines, such as TNF-α, IL-17, CXCL1, and CXCL2, in the hapten-challenged skin, resulting in the elicitation of CHS (Kish *et al.*, 2009). Therefore, it is possible that the increase in various cell populations in the ear skin of BTLA$^{-/-}$ mice (Figure 1c) as well as the reduction of various cell populations by 6A6 may be because of the alteration of the levels of CD8^{+} T-cell-derived IFN-γ.

We also found that although the number of CD4^{+} T cells in the draining lymph nodes of DNFB-sensitized BTLA$^{-/-}$ mice was similar to that of DNFB-sensitized WT mice (Figure 2a), the number of CD4^{+} T cells at the site of CHS was increased in BTLA$^{-/-}$ mice compared with WT mice (Figure 1c). In addition, we found that the expression of CXCR3, a chemokine receptor that is mainly expressed in Th1-type CD4^{+} T cells and effector CD8^{+} T cells and regulates their migration (Groom and Luster, 2011), on CD44^{high} LY6C^{low} CD4^{+} T cells was indistinguishable between BTLA$^{-/-}$ mice and WT mice (Figure 2c). These findings suggest that CXCR3 ligands may not be the major chemokines for the recruitment of CD4^{+} T cells at the site of CHS. Further studies are required to address the mechanism underlying the enhanced CD4^{+} T-cell infiltration at the site of CHS in BTLA$^{-/-}$ mice.

A previous study has shown that although BTLA is expressed at low levels in FOXP3^{+} regulatory T cells, FOXP3^{+} regulatory T cells in BTLA$^{-/-}$ mice exhibited normal suppressive activity (Tao *et al.*, 2008). In this study, we found that the frequency of FOXP3^{+} CD4^{+} T cells in the draining lymph nodes of DNFB-sensitized BTLA$^{-/-}$ mice was similar to that of DNFB-sensitized WT mice (Supplementary Figure S3a online). We also found that the frequency of ICOS^{+} CD25^{+} T cells, a subpopulation of regulatory T cells with strong suppressive activity on CHS (Vocanson *et al.*, 2010; Gomez de Agüero *et al.*, 2012), in the draining lymph nodes of DNFB-sensitized BTLA$^{-/-}$ mice was similar to that in DNFB-sensitized WT mice (Supplementary Figure S4 online). Furthermore, we found that the number and IL-10 production of FOXP3^{+} CD4^{+} T cells recovered from the ear skin in CHS were similar between WT mice and BTLA$^{-/-}$ mice (Supplementary Figure S5 online). Taken together, these results suggest that BTLA is not significantly involved in the regulation of FOXP3^{+} regulatory T cells in hapten-induced CHS.

Our experiments using an agonistic anti-BTLA mAb (6A6) have revealed that the agonistic agent for BTLA signaling has a therapeutic potential for CHS, even though the agent is administered to the sensitized mice at 24 hours after the challenge. As it has been shown that 6A6 does not perturb protective immunity against viral and bacterial infection (Albring *et al.*, 2010), it is suggested that agonistic agents for BTLA could suppress CHS without the risk of infection. Although agonistic anti-human BTLA antibody has not been developed yet, our data provide a rationale for the development of agonistic agents for the BTLA pathway for a number of CD8^{+} T-cell-mediated immune diseases, including allergic contact dermatitis.

The mode of action of 6A6 has been intensively examined by recent studies (Lepenies *et al.*, 2007; Albring *et al.*, 2010). It has been reported that 6A6 is indeed an agonistic antibody rather than depleting antibody for cells expressing BTLA (Lepenies *et al.*, 2007; Albring *et al.*, 2010). Consistent with these studies, we found that *in vivo* injection of 6A6 at 24 hours after DNFB challenge decreased the number of CD8^{+} T cells at the site of CHS (Figure 5) but not in the spleen (Supplementary Figure S7 online). We also found that 6A6 more strongly decreased the number of IFN-γ-producing CD8^{+} T cells at the site of CHS (Figure 5), further supporting the agonistic activity of 6A6 for BTLA. It has also been reported that 6A6 suppresses the function of BTLA-expressing cells even in herpesvirus entry mediator–deficient mice (Lepenies *et al.*, 2007; Albring *et al.*, 2010), suggesting that 6A6 activates BTLA signaling independent of herpesvirus entry mediator.

In conclusion, we show here that BTLA attenuates CHS presumably by suppressing hapten-induced activation of CD8^{+} T cells. Although further studies are required for understanding the basis of the findings, our results should highlight the therapeutic potential of agonistic agents for the BTLA pathway for CD8^{+} T-cell-mediated immune diseases, including allergic contact dermatitis.

MATERIALS AND METHODS

Mice

BTLA-deficient (BTLA$^{-/-}$) mice on BALB/c background and C57BL/6 background (Watanabe *et al.*, 2003) were bred and housed in the animal facility at Chiba University. SCID mice and C57BL/6 mice were purchased from Japan SLC (Shizuoka, Japan). All mice were housed in microisolator cages under specific pathogen-free conditions and all experiments were conducted according to the guidelines of Chiba University (approved ID; 21–11).

DNFB-induced CHS

Mice were sensitized on the shaved abdomen with 30 μl of 0.5% DNFB (Sigma-Aldrich, St Louis, MO) in acetone. Five days later, mice were painted with 0.2% DNFB in acetone to the right ear (10 μl to each side) and acetone alone (as a negative control) to the left ear. Ear swelling was quantified by three consecutive measurements of ear thickness using a caliper.

Histological analysis

Samples of the ear pinna were fixed in 10% buffered formalin and embedded in paraffin. Sections (4 μm in thickness) were stained with hematoxylin and eosin (HE).

Isolation of leukocytes from ear skin

The ear skin was cut into small pieces and incubated in RPMI 1640 medium containing Liberase TL (0.5 mg ml^{-1}, Roche, Basel, Switzerland) for 1 hour at 37°C. A suspension of single cells was separated from undigested tissue by passing through a 40-μm nylon cell strainer.

Flow cytometric analysis

Cells were stained and analyzed on a FACSCalibur (Becton Dickinson, San Jose, CA) using CELLQuest software. The following antibodies were purchased: anti-CD4 (RM4-5, BioLegend, San Diego, CA), anti-CD8α (53-6-7, BD Biosciences, San Jose, CA), anti-Gr-1 (RB6-8C5, BD Biosciences), anti-F4/80 (BM8, BioLegend), anti-BTLA (6F7, eBioscience, San Diego, CA), anti-CD107a (1D4B, BioLegend), anti-CD44 (IM7, BD Bioscience), anti-LY6C (AL-21, BD Bioscience), anti-FOXP3 (FJK-16s, eBioscience), anti-GITR (DTA-1, eBioscience), anti-CTLA4 (UC10-4B9, eBioscience), anti-CD25 (7D4, BD Bioscience), anti-CD207 (RMUL.2, eBioscience), anti-ICOS (C398.4A, eBioscience), and anti-hamster IgG cocktail (BD Bioscience). Before staining, Fc receptors were blocked with anti-CD16/32 antibody (2.4G2; BioLegend). Negative controls consisted of isotype-matched directly conjugated, nonspecific antibodies (BD Biosciences).

Intracellular staining for IFN-γ and IL-10

Cells recovered from the ear were incubated with monensin (2 μM) (Sigma-Aldrich) in RPMI1640 medium for 5 hours, and intracellular staining for IFN-γ and IL-10 was performed using anti-IFN-γ PE (XMG1.2; BD Biosciences) and anti-IL-10 antigen-presenting cell (JES5-16E3; BD Bioscience) as described previously (Suto *et al.*, 2008).

Cell transfer experiments

$CD4^+$ T cells and $CD8^+$ T cells were isolated from the spleen of WT mice or $BTLA^{-/-}$ mice by using PE-labeled anti-mouse CD4 antibody (eBioscience) and anti-mouse CD8 antibody (eBioscience), respectively, and magnetic microbeads were coated with anti-PE antibody (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions. Mixture of splenic $CD4^+$ T cells (1×10^6 cells) and $CD8^+$ T cells (1×10^6 cells) from either WT mice or $BTLA^{-/-}$ mice were transferred intravenously to SCID mice (7-weeks-old). The recipient mice were sensitized and challenged with DNFB as described above.

The effect of an agonistic anti-BTLA antibody on CHS

C57BL/6 mice were sensitized on the shaved abdomen with DNFB, and 5 days later, mice were challenged with DNFB as described above. Either just before or 24 hours after DNFB challenge, 200 μg of an agonistic anti-BTLA mAb (clone 6A6, hamster IgG) (Lepenies *et al.*, 2007) or control IgG was injected intraperitoneally to the mice. Ear swelling and infiltrating leukocytes in the ear skin were evaluated as described above.

Proliferation assay

WT mice and $BTLA^{-/-}$ mice were sensitized with 0.5% DNFB on arms and legs (30 μl each). Five days after the sensitization, single-cell suspensions were prepared from inguinal and axillary LNs, and $CD4^+$ T cells and $CD8^+$ T cells were isolated by magnetic cell sorting as described above. $CD62L^{low}$ $CD4^+$ T cells and $CD62L^{low}$ $CD8^+$ T cells were purified from isolated $CD4^+$ T cells and $CD8^+$ T cells, respectively, by eliminating $CD62L^{high}$ cells with anti-CD62L antibody–conjugated microbeads (Miltenyi Biotec) according to the manufacturer's instructions. Purified $CD62L^{low}$ $CD4^+$ T cells or $CD62L^{low}$ $CD8^+$ T cells (2×10^5 cells per well) were stimulated with DNBS (100 μg ml^{-1}, Sigma-Aldrich) in the presence of irradiated splenocytes (1×10^6 cells per well) as antigen-presenting cells in a 96-well plate for 3 days. Cell proliferation was assessed using Premix WST-1 Cell Proliferation Assay System (Takara Bio, Shiga, Japan).

Measurement of cytokines

Isolated $CD62L^{low}$ $CD4^+$ T cells or $CD62L^{low}$ $CD8^+$ T cells were stimulated with DNBS in the presence of irradiated splenocytes for 3 days as described above. The amounts of IFN-γ and IL-4 in the culture supernatants were measured by using murine ELISA kits for IFN-γ and IL-4, respectively (BD Biosciences). The assay was performed in duplicate according to the manufacturer's instructions. The detection limits of these assays were 15 pg ml^{-1}.

Data analysis

Data are summarized as mean ± SD. The statistical analysis of the results was performed by means of ANOVA or unpaired *t*-test as appropriate. *P*-values <0.05 were considered significant.

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 http://www.nature.com/jid

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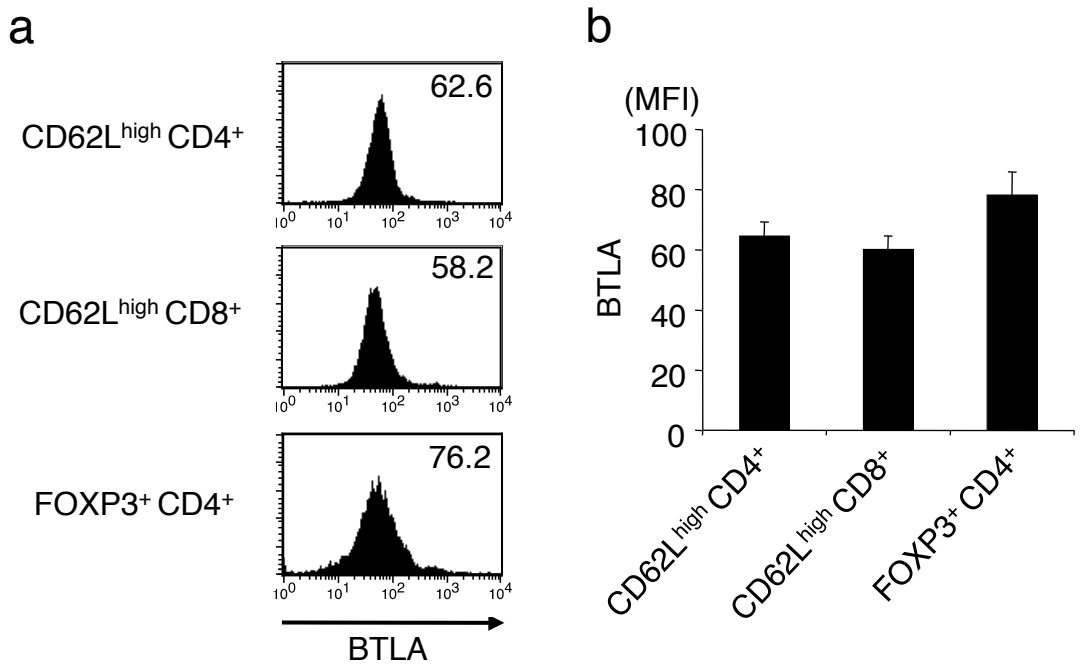
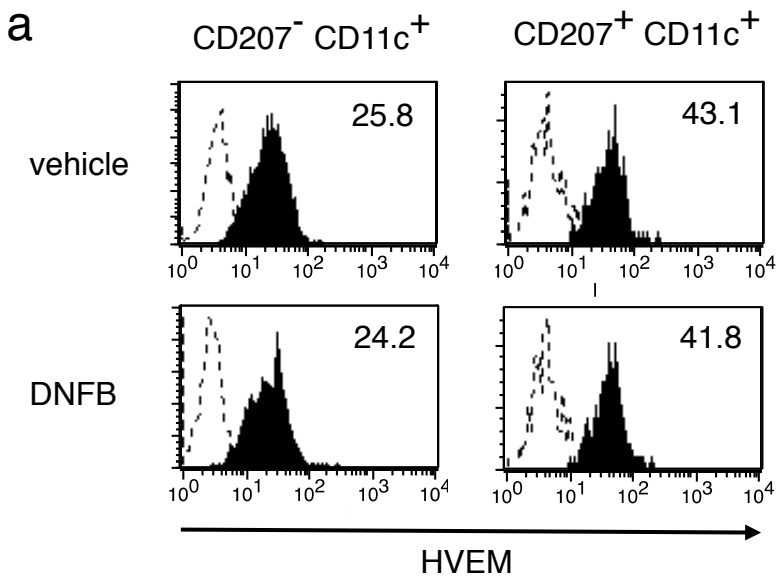
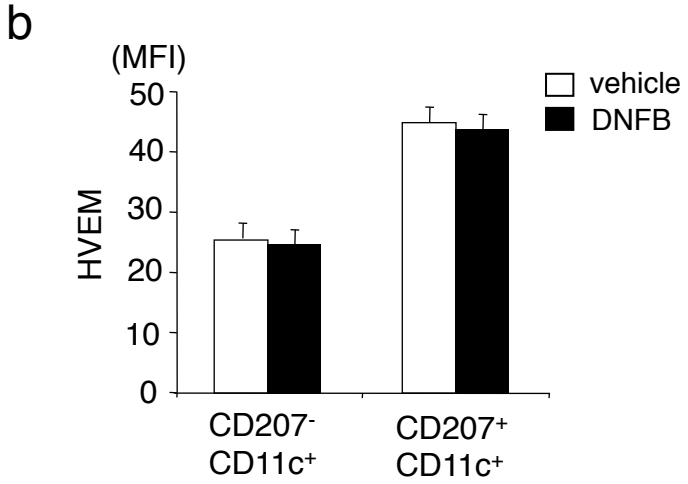


Supplementary figure 1. BTLA is similarly expressed in naive CD4$^+$ T cells, CD8$^+$ T cells, and FOXP3$^+$CD4$^+$ T cells.

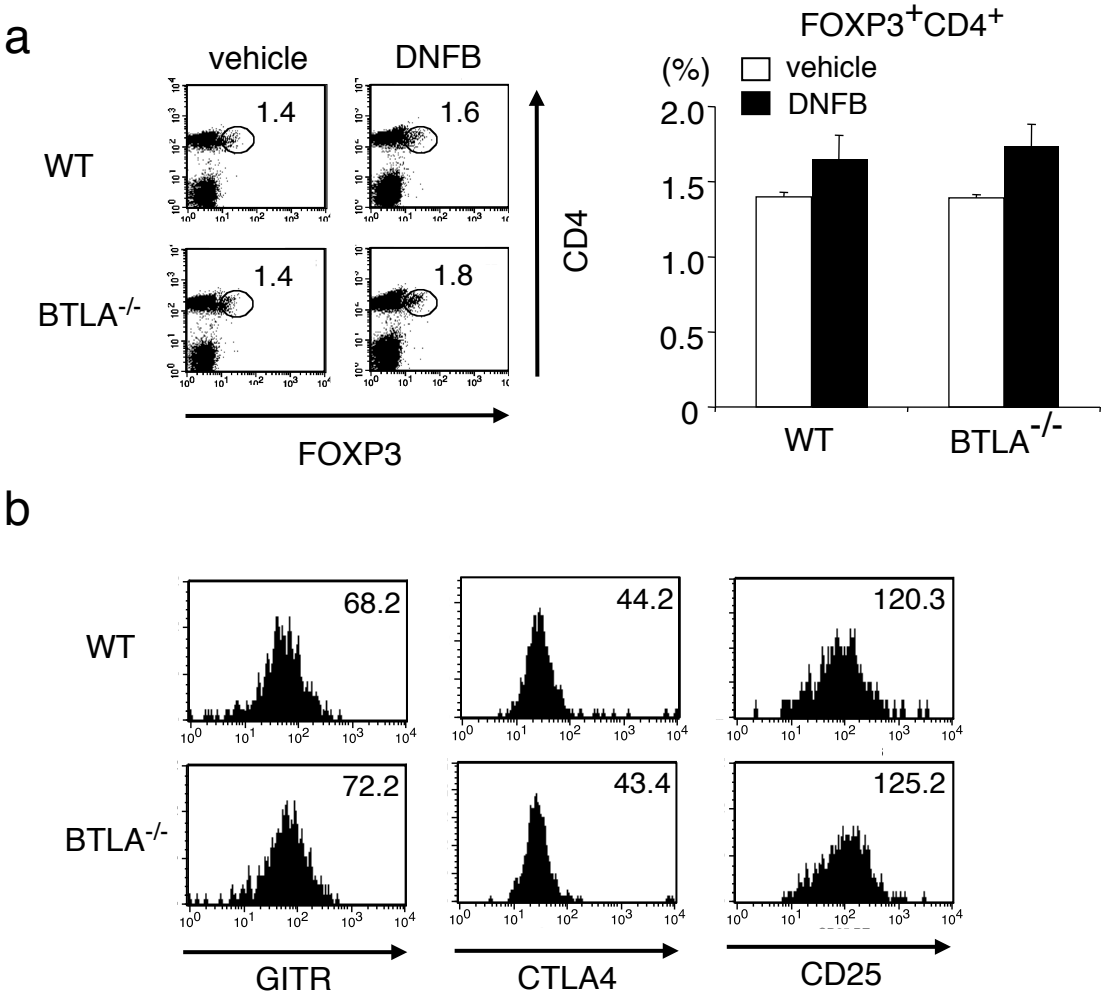
(**a, b**) Cells were prepared from draining lymph nodes at 5 days after sensitization with DNFB and the expression levels of BTLA on CD62L^{high} CD4$^+$ cells, CD62L^{high} CD8$^+$ cells, and FOXP3$^+$ CD4$^+$ cells were analyzed by flow cytometry. Shown are representative FACS profiles (**a**) and means ± SD (n = 5, each) of the mean fluorescence intensity (MFI) of BTLA expression (**b**). Data are representative of three independent experiments.





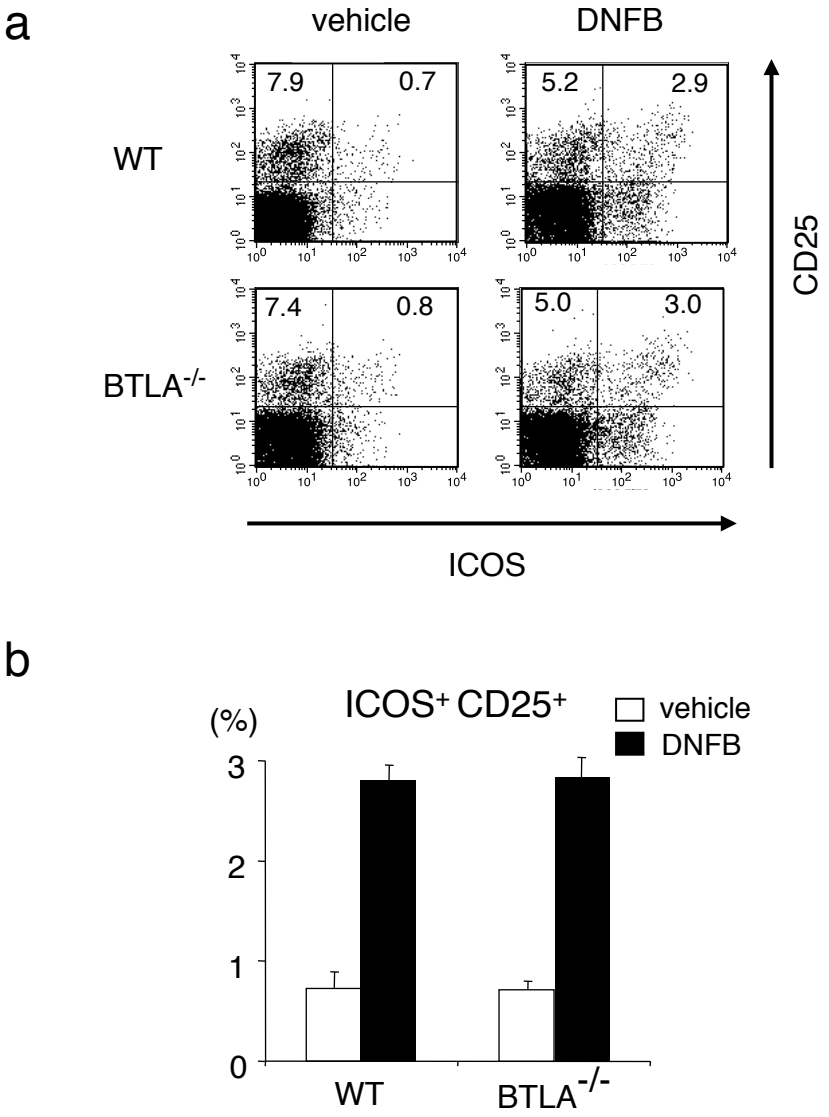
Supplementary figure 2. DNFB challenge does not alter the expression levels of HVEM on dermal dendritic cells and Langerhans cells.

(**a, b**) C57BL/6 mice were sensitized with DNFB and 5 days later, mice were challenged with DNFB on the ear. Two days after the challenge, cells were isolated from the ear and analyzed on flow cytometry. Shown are representative histograms of HVEM staining (solid line) with isotype matched control staining (dotted line) (**a**) and means ± SD (n = 5, each) of MFI of HVEM staining (**b**) on $CD207^{-}CD11c^{+}$ cells (dermal dendritic cells) and $CD207^{+}CD11c^{+}$ cells (Langerhans cells). Data are representative of two independent experiments.



Supplementary figure 3. The frequency and cell surface markers of FOXP3^{+} CD4^{+} T cells in the draining lymph nodes of DNFB-sensitized BTLA$^{-/-}$ mice are similar to those in DNFB-sensitized WT mice.

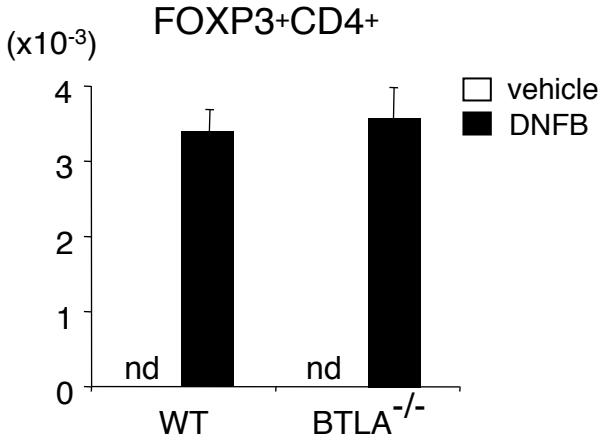
BTLA$^{-/-}$ mice and littermate WT mice were sensitized with DNFB on the arms and legs as described in the Materials and Methods. Five days after the sensitization, cells from inguinal and axillary lymph nodes were analyzed on flow cytometry. (**a**) Shown are representative dot plots of CD4 vs. FOXP3 staining and means ± SD (n = 5) of the frequency of FOXP3^{+} CD4^{+} cells. (**b**) Shown are representative histograms of GITR, CTLA4, and CD25 on FOXP3^{+} CD4^{+} T cells. Data are representative of three independent experiments.



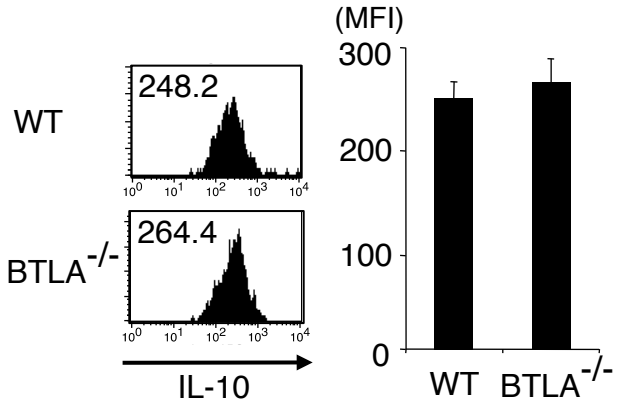
Supplementary figure 4. The frequency of ICOS^{+} regulatory T cells in the draining lymph nodes of DNFB-sensitized BTLA$^{-/-}$ mice is similar to that in DNFB-sensitized WT mice.

BTLA$^{-/-}$ mice and littermate WT mice were sensitized with DNFB on the arms and legs as described in the Materials and Methods. Five days after the sensitization, cells from inguinal and axillary lymph nodes were analyzed on flow cytometry. (**a**) Shown are representative dot plots of CD25 vs. ICOS staining of CD4^{+} T cells and (**b**) means ± SD (n = 5) of the frequency of ICOS^{+} CD25^{+} cells in CD4^{+} T cells. Data are representative of two independent experiments.

a

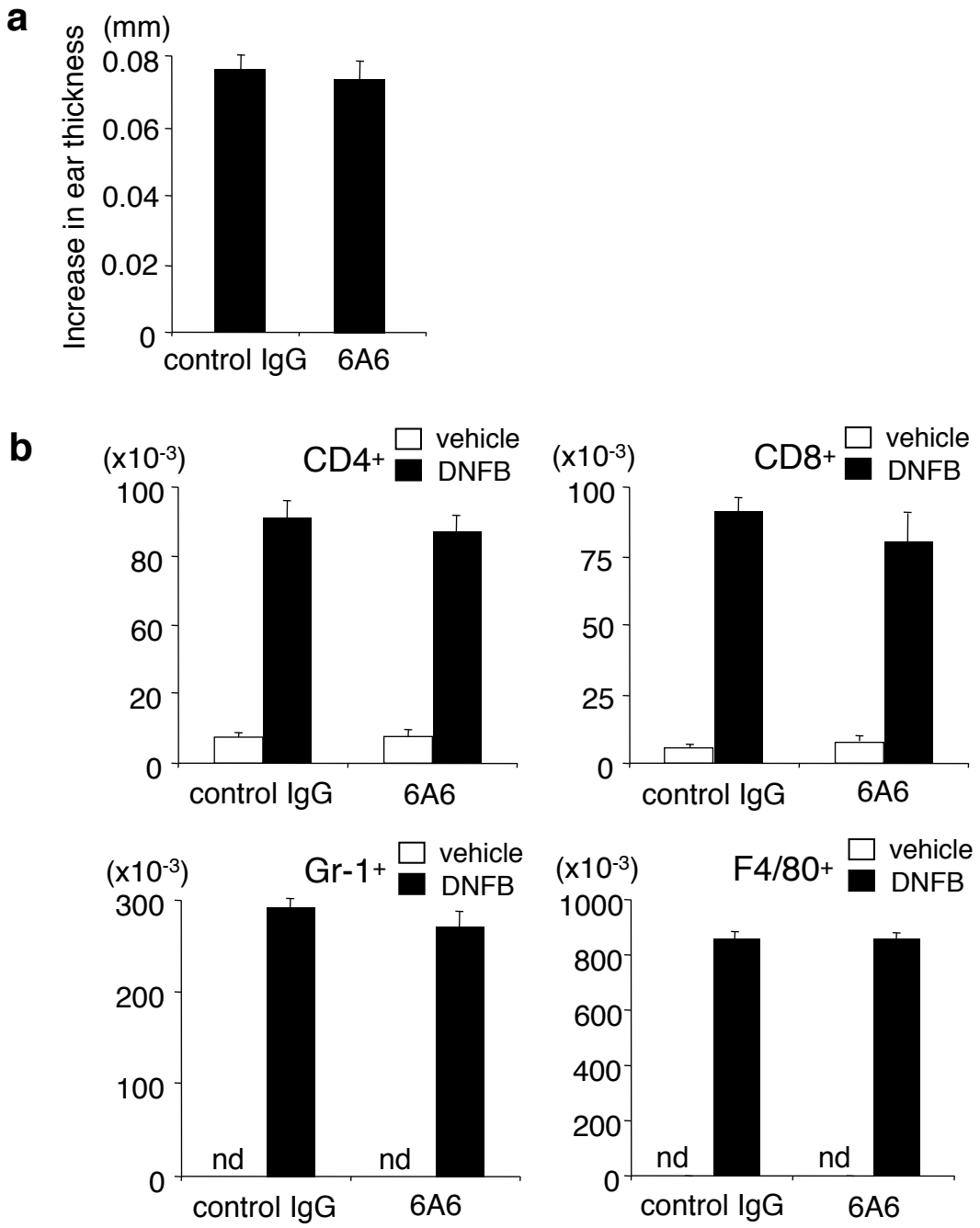


b



Supplementary figure 5. The number of FOXP3$^+$ CD4$^+$ T cells and IL-10 production in FOXP3$^+$ CD4$^+$ T cells are indistinguishable between WT mice and BTLA$^{-/-}$ mice.

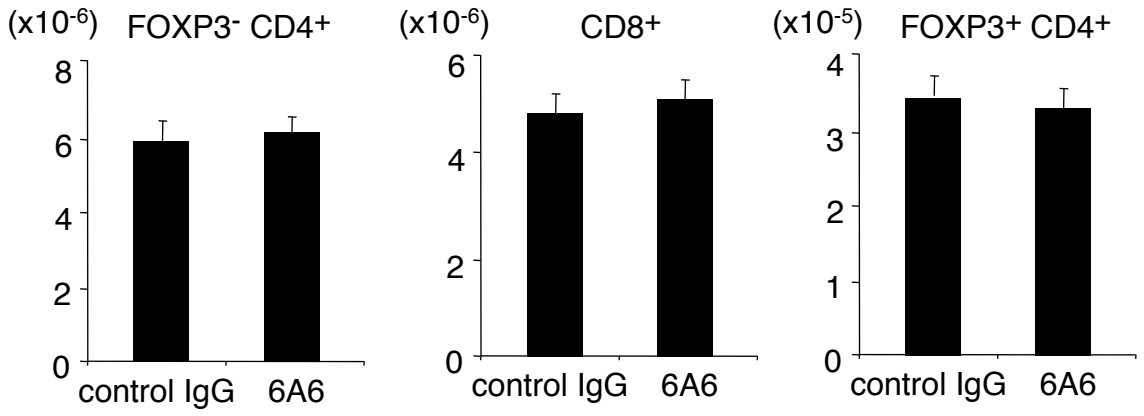
(**a, b**) BTLA$^{-/-}$ mice and littermate WT mice were sensitized with DNFB and 5 days later, mice were challenged with DNFB on the ear. Two days after the challenge, cells were isolated from the ear and analyzed on flow cytometry. (**a**) Shown are means ± SD (n=5) of the numbers of FOXP3$^+$ CD4$^+$ T cells. nd = not detectable. (**b**) The expression of IL-10 in FOXP3$^+$ CD4$^+$ T cells isolated from the ear was examined by flow cytometry. Shown are representative histograms with mean fluorescence intensity (MFI) and means ± SD (n = 5, each) of the MFI of anti-IL-10 staining. Data are representative of two independent experiments.



Supplementary figure 6. In vivo injection of an agonistic anti-BTLA mAb does not inhibit CHS in BTLA$^{-/-}$ mice.

(**a, b**) BTLA$^{-/-}$ mice on C57BL/6 background were sensitized with DNFB and 5 days later, mice were injected intraperitoneally with an agonistic anti-BTLA mAb (6A6) or control IgG and then challenged with DNFB on the ear. Two days after the challenge, ear swelling (**a**) as well as the numbers of CD4^{+} T cells, CD8^{+} T cells, Gr-1^{+} cells, and F4/80^{+} cells isolated from the ear (**b**) were evaluated. Shown are means ± SD (n = 5 mice in each group). nd = not detectable. Data are representative of two independent experiments.

Supplementary figure 7



Supplementary figure 7. In vivo injection of an agonistic anti-BTLA mAb does not deplete FOXP3$^-$ CD4$^+$ T cells, CD8$^+$ T cells, or FOXP3$^+$ CD4$^+$ T cells.
C57BL/6 mice were sensitized with DNFB and 5 days later, mice were injected intraperitoneally with 6A6 or control IgG and then challenged with DNFB on the ear. Two days after the challenge, the numbers of FOXP3$^-$ CD4$^+$ T cells, CD8$^+$ T cells, and FOXP3$^+$ CD4$^+$ T cells in the spleen were evaluated by flow cytometry. Shown are means ± SD (n = 5 mice in each group). Data are representative of two independent experiments.