It has long been proposed that various aspects of immune responses are regulated by activities of the nervous system (Elenkov et al., 2000; Bellinger et al., 2008). However, the cellular and molecular basis for neural regulation of immunity has emerged over the past decade (Andersson and Tracey, 2012; Scheiermann et al., 2013; Curtis et al., 2014). Recently, roles of adrenergic nerves in the regulation of immune cell dynamics were demonstrated. Adrenergic nerves controlled the recruitment of myeloid cells into tissues by establishing circadian oscillations of adhesion molecule and chemokine expression by vascular endothelial cells (Scheiermann et al., 2012). Another study demonstrated that elevated sympathetic activity after stroke induced behavioral changes of invariant natural killer T cells in the liver through β2-adrenergic receptors expressed on their surface (Wong et al., 2011). However, although blood lymphocyte numbers exhibit circadian oscillations (Scheiermann et al., 2012), it remains unclear how the inputs from adrenergic nerves affect the trafficking of B and T cells, major subsets of lymphocytes involved in adaptive immune responses.

Blood lymphocyte numbers are maintained by recirculation through secondary lymphoid organs. After entering a LN from blood, lymphocytes travel to separate subcompartments, where they survey for antigen. After spending several hours to a day in the LN, lymphocytes exit into lymph and eventually return to the bloodstream through the thoracic duct, which allows lymphocytes to continue antigen surveillance. Among these events, egress from LNs is critical for the regulation of lymphocyte recirculation (Cyster and Schwab, 2012). Lymphocyte egress from LNs is dependent on sphingosine-1-phosphate receptor-1 (S1PR1), by which lymphocytes sense S1P gradients between lymph (~100 nM) and LN parenchyma (~1 nM) to exit LNs. S1PR1 acts to overcome...
retention signals mediated by the chemokine receptor CCR7 and other Gαi-coupled receptors (Pham et al., 2008). Thus, the rate of lymphocyte egress from LNs appears to be determined by the relative strength of egress-promoting signals versus retention-promoting signals.

It has been established that pharmacological modulation of lymphocyte trafficking is effective for the treatment of autoimmune diseases (Steinman, 2014). The functional S1PR1 antagonist FTY720 (Fingolimod/Gilenya), which causes down-modulation of S1PR1 (Rosen and Goetzl, 2005; Schwab and Cyster, 2007), is approved for the treatment of multiple sclerosis. A major proposed action of FTY720 is to inhibit LN egress of autoreactive T cells and consequently their invasion into inflammatory sites (Brinkmann et al., 2010). Thus, lymphocyte egress from LNs represents an important point of regulation in the pathology of immune disorders.

Here, we report that inputs through lymphocyte β2-adrenergic receptors (β2ARs), which are at least in part provided by adrenergic nerves, enhance signals through the retention-promoting chemokine receptors and consequently inhibit lymphocyte egress from LNs. In the context of T cell-mediated inflammation, we show that activation of β2ARs sequesters antigen-primed T cells in LNs and prevents their migration to inflamed tissues, suggesting a mechanism for β2AR-mediated suppression of inflammatory responses.

RESULTS
Stimulation of β2ARs causes lymphopenia by a cell-intrinsic mechanism

Because β2ARs are predominantly expressed in lymphocytes compared with other subtypes of adrenergic receptors (Sanders, 2012), we treated mice with selective β2AR agonists, clenbuterol, or salbutamol, to mimic activation of adrenergic nerves and test the possible role of β2ARs in lymphocyte dynamics. Administration of a single dose of either β2AR agonist resulted in a rapid reduction of blood B cells and CD4+ or CD8+ T cells in a dose-dependent manner (Fig. 1 A; Fig. S1 A; and not depicted). Notably, the reduction of blood lymphocytes was accompanied by a sharp decline of lymphocyte numbers in lymph (Fig. 1 B and Fig. S1 B). Consistent with the reported pharmacological properties of β2AR agonists (Smith, 1998), clenbuterol was more potent than salbutamol. The ED50 values of clenbuterol and salbutamol for decreasing circulating B cell numbers were 0.01 and 0.3 mg/kg, respectively, which were comparable to those reported for rodents (McElroy and O’Donnell, 1988). Although the lymphocyte numbers after administration of either β2AR agonist exhibited a trough at 2 h (Fig. 1, C and D), their recovery was faster in salbutamol-treated mice, reflecting a shorter half-life of salbutamol compared with clenbuterol (Smith, 1998). Lymphopenia produced by β2AR stimulation was prominent in B cells compared with T cells, which was consistent with the predominant expression of transcripts for β2ARs in B cells (Fig. 1 E and Fig. S1, C and D). Flow cytometric analysis of the surface expression of β2ARs was hindered by lack of an antibody to produce specific labeling.

The effect of clenbuterol on the numbers of circulating B and T cells was abrogated in β2AR-deficient mice (Fig. 1, F and G; and not depicted), confirming that clenbuterol specifically acts on β2ARs in this experimental setting. To determine whether the action of the β2AR agonist was mediated by β2ARs expressed on the radiosensitive hematopoietic compartment or radioresistant nonhematopoietic compartment, we administered clenbuterol to BM chimeras generated using β2AR-deficient mice as donors or recipients. Reduction of B cell numbers in blood and lymph was significantly blunted, but not completely abrogated, in WT mice reconstituted with β2AR-deficient BM cells (Fig. 1, H and I). In contrast, B cell numbers in β2AR-deficient mice receiving WT BM cells were reduced to the same extent as WT recipients (Fig. 1, H and I). The same was true of T cells in the BM chimeras (unpublished data). These observations suggest that lymphopenia induced by treatment with the β2AR agonist largely depends on β2ARs expressed on hematopoietic cells although the involvement of nonhematopoietic β2ARs cannot be excluded.

Lymphocyte egress from LNs is inhibited by β2AR stimulation

Because the reduction of lymphocytes in lymph as well as blood was reminiscent of changes induced by FTY720 treatment (Mandala et al., 2002), we hypothesized that stimulation of β2ARs might inhibit lymphocyte egress from LNs. To test this possibility, we implanted osmotic pumps in mice, allowing continuous administration of clenbuterol or salbutamol, and then blocked lymphocyte entry to LNs by treatment with neutralizing antibodies against α4 and αL integrins for 22 h (Fig. 2 A; Lo et al., 2005). Treatment with either β2AR agonist increased the numbers of B cells and CD4+ or CD8+ T cells that were retained in the mesenteric LNs after entry blockade in a dose-dependent manner (Fig. 2 B; Fig. S2 A; and not depicted), suggesting that β2AR stimulation retards lymphocyte egress from LNs. Consistent with the effect on circulating lymphocyte numbers, higher doses of salbutamol were required to achieve the levels of egress inhibition by clenbuterol. On the other hand, treatment with the β2AR agonists did not affect lymphocyte entry to LNs (Fig. 2 C and Fig. S2 B), suggesting their selective action on lymphocyte egress. In WT mice reconstituted with β2AR-deficient BM cells, LN retention of B and T cells was not promoted by clenbuterol treatment (Fig. 2 D and not depicted), whereas β2AR stimulation resulted in enhanced lymphocyte retention in the LNs of β2AR-deficient mice reconstituted with WT BM cells (Fig. 2 E and not depicted). These observations indicate that stimulation of β2ARs inhibits egress of lymphocytes from LNs in a cell-intrinsic manner.

Physiological inputs through β2ARs contribute to lymphocyte retention in LNs

There was a trend for β2AR-deficient mice to have higher lymphocyte numbers in both blood and lymph compared with WT mice (Fig. 1, F and G), suggesting that physiological adrenergic stimuli through β2ARs are involved in lymphocyte
β2AR-mediated signals act to limit lymphocyte egress from LNs in a cell-intrinsic manner. However, such biased distribution was not observed for CD4+ or CD8+ T cells (unpublished data). Because of the lower expression of, and thereby weaker signals through β2ARs in T cells compared with B cells (Fig. 1E), the difference in LN egress between WT and mutant T cells might have been compensated for during long-term BM reconstitution.

To directly address the role of lymphocyte β2ARs in LN egress under physiological conditions, we performed short-term recirculation. To test the contribution of β2ARs expressed on hematopoietic cells, we generated mixed BM chimeras using a mixture of CD45.2+ β2AR-deficient and CD45.1+ WT cells, and examined lymphocyte distribution in lymphoid tissues. Compared with their frequencies in mesenteric LNs, blood and lymph showed a significant accumulation of β2AR-deficient B cells over WT B cells (Fig. 3A and Fig. S3A). This accumulation was not observed in control chimeras generated using a mixture of congenically distinct β2AR-sufficient and WT BM cells. These observations support that β2AR stimulation induces lymphopenia in a cell-intrinsic manner. (A and B) Numbers of B cells (CD19+, top) and CD4+ T cells (CD4+, bottom) in blood (A) and lymph (B) that were collected from WT mice 2 h after i.v. injection with the indicated doses of clenbuterol or salbutamol. Data are pooled from seven experiments and are shown as mean ± SEM for at least three mice. (C and D) Numbers of B cells (top) and CD4+ T cells (bottom) in blood (C) and lymph (D) at the indicated times after i.v. injection of saline, clenbuterol (0.4 mg/kg), or salbutamol (12 mg/kg). Data are pooled from 11 experiments and are shown as mean ± SEM for at least five mice. (E) Quantitative RT-PCR analysis showing the abundance of Adrb2 mRNA transcripts (encoding β2AR) relative to Gapdh (encoding glyceraldehyde-3-phosphate dehydrogenase) for the indicated lymphocyte populations isolated from the mouse spleen. Data are representative of three experiments and shown as mean ± SD of triplicates. (F and G) B cell numbers in blood (F) and lymph (G) at the indicated times after i.v. injection of saline, clenbuterol (0.4 mg/kg), and 2 h later, the fractions of B cells in the blood (H) and lymph (I) were determined as percentages relative to those in saline-treated mice. Data are pooled from six experiments and the dashed lines show the levels of saline-treated control as 100%. Each symbol represents an individual mouse and bars indicate means (F–I). Sal, Saline; Clen, clenbuterol; Salb, salbutamol. *, P < 0.05; **, P < 0.01; ****, P < 0.0001; ns, not significant (one-way ANOVA with Bonferroni’s post-test).
Lymphocyte numbers in mesenteric LNs were underestimated in 6-OHDA–treated mice compared with control mice (Fig. 3 D), suggesting that inputs from adrenergic nerves retard lymphocyte egress from LNs. However, the contribution of humoral adrenergic inputs provided by adrenal glands cannot be excluded. Collectively, these findings indicate that physiological levels of inputs through $\beta_2$ARs, a part of which is provided by adrenergic nerves, help retain lymphocytes within LNs by limiting their egress and maintain homeostasis of their distribution among lymphoid tissues.

**Functional and physical association of $\beta_2$ARs with retention-promoting chemokine receptors**

A recent study demonstrated that CXCR4-deficient B cells exited LNs more rapidly than WT B cells (Schmidt et al., 2013), suggesting that CXCR4, in addition to CCR7, might promote lymphocyte retention in LNs by counteracting the egress-promoting receptor S1PR1. Thus, we anticipated that stimulation of lymphocyte $\beta_2$ARs might modulate lymphocyte egress from LNs. During continuous administration of saline or $\beta_2$AR agonists, WT mice were treated with neutralizing antibodies against $\alpha_4$ and $\alpha_\text{L}$ integrins for 22 h. Fractions of lymphocytes remaining in LNs after integrin blockade were determined as ratios relative to those in saline-treated mice that did not undergo integrin blockade. (B) Fractions of CD4+ T cells (CD4+) and B cells (CD19lgD$^+$CD95$^-$) remaining in mesenteric LNs were determined upon treatment with saline or the indicated doses of clenbuterol or salbutamol. Data are shown as mean ± SEM for at least three mice. (C) CD45.1+ WT spleen cells were transferred into WT hosts, allowed to equilibrate for 2 d, and further lymphocyte entry to LNs was blocked by integrin-neutralizing antibodies for 14 h. $\beta_2$AR-deficient lymphocytes were retained in the mesenteric LNs to a lesser extent than $\beta_2$AR-sufficient lymphocytes (Fig. 3 B and Fig. S3 B). This result confirms that physiological inputs through $\beta_2$ARs expressed on lymphocytes inhibit their egress from LNs. Consistent with unaffected lymphocyte homing by agonist stimulation of $\beta_2$ARs (Fig. 2 C), transferred CD45.2+ $\beta_2$AR-deficient lymphocytes entered LNs as efficiently as CD45.1+ WT lymphocytes (Fig. 3 C and Fig. S3 C), further ruling out the involvement of $\beta_2$ARs in lymphocyte entry to LNs.

To evaluate the contribution of adrenergic nerves in controlling lymphocyte egress from LNs, we treated mice with 6-hydroxydopamine (6-OHDA) to deplete peripheral neuronal terminals containing noradrenaline, and then performed entry blockade with integrin-neutralizing antibodies for 14 h. Lymphocyte numbers in mesenteric LNs were underrepresented in 6-OHDA–treated mice compared with control mice (Fig. 3 D), suggesting that inputs from adrenergic nerves retard lymphocyte egress from LNs. However, the contribution of humoral adrenergic inputs provided by adrenal glands cannot be excluded. Collectively, these findings indicate that physiological levels of inputs through $\beta_2$ARs, a part of which is provided by adrenergic nerves, help retain lymphocytes within LNs by limiting their egress and maintain homeostasis of their distribution among lymphoid tissues.

**Figure 2. $\beta_2$AR stimulation inhibits lymphocyte egress from LNs.** (A) Experimental design for $\beta_2$AR agonist administration and integrin blockade. During continuous administration of saline or $\beta_2$AR agonists, WT mice were treated with neutralizing antibodies against $\alpha_4$ and $\alpha_\text{L}$ integrins for 22 h.

Fractions of lymphocytes remaining in LNs after integrin blockade were determined as ratios relative to those in saline-treated mice that did not undergo integrin blockade. (B) Fractions of CD4+ T cells (CD4+) and B cells (CD19lgD$^+$CD95$^-$) remaining in mesenteric LNs were determined upon treatment with saline or the indicated doses of clenbuterol or salbutamol. Data are shown as mean ± SEM for at least three mice. (C) CD45.1+ WT spleen cells were transferred into WT mice that had been treated with saline, clenbuterol (0.4 mg/kg), or salbutamol (12 mg/kg) and enumerated in mesenteric LNs 90 min after transfer. Data are shown as the number of CD4+ T cells (CD4+) or B cells (CD19+) recovered in each recipient per $10^6$ transferred CD45.1+ populations. (D and E) BM chimeras generated using WT or $\textit{Adrb}^{-/-}$ mice as donors (D) or recipients (E) were treated as in A. The remaining fraction of B cells (CD19lgD$^+$CD95$^-$) is shown for each. Each symbol represents an individual mouse and bars indicate means (C–E). Data are pooled from two (B and E) or three (C and D) experiments. Sal, saline; Cen, clenbuterol; Salb, salbutamol. **, P < 0.01; ns, not significant. P-values were obtained by one-way ANOVA with Bonferroni’s post-test (C) or unpaired Student’s t test (D and E).
The β2AR agonist in the absence of chemoattractants did not induce activation of Rac1 in lymphocytes (Fig. 4 C). These observations suggest that stimulation of β2ARs preferentially enhances signals mediated by the retention-promoting chemokine receptors CCR7 and CXCR4.

To examine whether the enhanced signals affect lymphocyte chemotaxis, we tested Transwell migration in the presence of the β2AR agonist using β2AR-deficient lymphocytes as a reference. We used suboptimal concentrations of chemoattractants to favor the likelihood that possible chemotaxis-enhancing effects of β2AR stimulation, if any, could be detected. Consistent with the results in Rac1 activation assays, B cells exhibited enhanced chemotactic responses mediated by CCR7 or CXCR4, but not S1PR1 or CXCR5 (Fig. 4 D and Fig. S4). In CD4+ or CD8+ T cells, enhancement of Transwell migration by β2AR stimulation was detectable only in response to
Figure 4. Functional and physical interactions of β2-ARs with chemokine receptors. (A and B) Activation of Rac1 was analyzed by Western blotting in B and T cells isolated from spleens of WT (A) or Adrb2−/− (B) mice at the indicated times after stimulation with CCL21 (2 µg/ml), CXCL12 (100 ng/ml), CXCL13 (1 µg/ml), or S1P (100 nM) in the presence or absence of clenbuterol (10 µM). (C) Rac1 activation was determined in B and T cells treated with clenbuterol in the absence of chemoattractants. Ratios of band density of GTP-bound Rac1 relative to that of total Rac1 were calculated and normalized to the ratio of the unstimulated (0 s) sample. The normalized ratios are shown under the lanes. Data are representative of more than two experiments for each cell type and condition. (D and E) Transwell migration of WT and Adrb2−/− splenic B cells (CD19+) and CD4+ T cells (CD4+) in response to CCL21 (100 ng/ml), CXCL12 (10 ng/ml), CXCL13 (300 ng/ml), or S1P (100 nM), plus clenbuterol (at the indicated concentrations). Dashed lines indicate the levels of migration of WT (red) and Adrb2−/− (blue) cells in the absence of chemoattractants and clenbuterol. Data are shown as mean ± range of duplicate
the CXCR4 ligand (Fig. 4 E and not depicted). The β2AR agonist alone did not induce Transwell migration of lymphocytes (unpublished data). Unaltered CCR7-mediated chemotaxis in T cells is consistent with a previous observation that transgenic overexpression of CCR7 did not enhance Transwell migration of mature T cells in response to CCR7 ligands (Kwan and Killeen, 2004). Because T cells show substantially higher chemotactic responses to CCR7 ligands compared with B cells (Ngo et al., 1998), we speculate that the effect of β2AR stimulation might be negated by the robust CCR7 responsiveness in T cells. Surface levels of CCR7 and CXCR4 on lymphocytes were not affected by agonist stimulation or deficiency of β2ARs (unpublished data). Overall, these findings suggest that β2AR stimulation enhances responsiveness of CCR7 and CXCR4 in lymphocytes.

Studies showed that different types of G protein–coupled receptors (GPCRs) form heteromeric complexes on the cell surface and cross-regulate their signaling (González-Maeso et al., 2008; Fribourg et al., 2011). These findings prompted us to test whether there is a physical association between β2ARs and CCR7 or CXCR4. Confocal microscopy for the surface localization of epitope-tagged receptors expressed in 2PK-3 B lymphoma cells demonstrated that β2ARs co-localized more frequently with CCR7 or CXCR4 compared with S1PR1 (Fig. 4 F), suggesting that β2ARs resides in the proximity of CCR7 or CXCR4. To examine whether the β2ARs form complexes with CCR7 or CXCR4, we performed co-immunoprecipitation assays. Using lysates prepared from transduced 2PK-3 cells, we found that β2ARs were co-precipitated with CCR7 or CXCR4, but not S1PR1 or CXCR5 (Fig. 4 G). Although we cannot exclude the involvement of additional molecules, this result provides evidence for physical interactions of β2ARs with CCR7 or CXCR4. However, analysis of these receptor interactions in mouse primary lymphocytes was not successful because of the lack of available specific antibodies for immunoprecipitation or immunoblotting of β2ARs. Collectively, these findings indicate that β2ARs are physically associated with CCR7 or CXCR4, which might contribute to the preferential enhancement of their signals by β2AR activation.

Enhanced lymphocyte retention by β2AR stimulation depends on CCR7 or CXCR4

The aforementioned findings suggest that stimulation of β2ARs inhibits lymphocyte egress from LN as a consequence of enhanced lymphocyte retention through CCR7 and CXCR4. To test the involvement of CCR7 in β2AR-mediated promotion of lymphocyte retention, we transferred CCR7-sufficient or -deficient spleen cells into WT mice and performed entry blockade with integrin-neutralizing antibodies for 18 h during β2AR stimulation with clenbuterol. Treatment with the β2AR agonist promoted LN retention of CCR7-sufficient and -deficient CD4+ or CD8+ T cells to the same extent (Fig. 5 A; Fig. S5; and not depicted). In contrast, enhancement of B cell retention was blunted, but not completely abrogated, by CCR7 deficiency (Fig. 5 B), suggesting that B cells are at least in part dependent on CCR7 in β2AR-mediated promotion of LN retention. A previous study showed that CCR7 deficiency resulted in more rapid LN egress of T cells, but not B cells (Pham et al., 2008), highlighting the retention-promoting role of CCR7 in T cells. However, our data suggest that when CCR7-mediated signals were increased from the basal level, they might also promote LN retention of B cells.

To test the involvement of CXCR4 in LN retention of T cells and confirm a role for B cell retention, we blocked CXCR4 function using a specific antagonist AMD3100 and analyzed the dynamics of lymphocytes. Treatment with AMD3100 increased the numbers of CD4+ T cells and B cells in blood and lymph (Fig. 5, C and D). Although part of the large increase of blood B cells might reflect the role of CXCR4 in their retention within BM (Ma et al., 1999), these data suggest that CXCR4 is involved in LN retention of both B and T cells. To test this, we performed entry blockade for 18 h during the continuous administration of AMD3100 through osmotic pumps. CD4+ T cells and B cells were underrepresented in the mesenteric LNs of mice treated with AMD3100 (40 mg/kg/d) compared with control mice (Fig. 5 E), confirming that CXCR4 functions as a retention-promoting receptor for both lymphocyte populations. Because a similar effect was observed with low (20 mg/kg/d) and high (80 mg/kg/d) doses of AMD3100 (unpublished data), we considered that complete blockade of CXCR4 was achieved with 40 mg/kg/d of the drug.

To assess the effect of CXCR4 blockade on β2AR- mediated enhancement of lymphocyte retention in LNs, we performed entry blockade upon combined treatment with AMD3100 and clenbuterol. Enhancement of T cell retention in LNs was abrogated by blockade of CXCR4 (Fig. 5 F), whereas enhancement of B cell retention was unaffected (Fig. 5 G). These results suggest that enhanced LN retention of T cells by β2AR stimulation is largely dependent on CXCR4. Thus, although we cannot exclude the involvement of other undefined retention-promoting receptors, β2AR–mediated enhancement of LN retention of B and T cells appears to be dependent on CCR7 and CXCR4, respectively.
Adrenergic control of lymphocyte egress | Nakai et al.

Figure 5. **β₂AR-mediated lymphocyte retention depends on coupled chemokine receptors.** (A and B) CFSE-labeled Ccr7⁺/⁺ or Ccr7⁻/⁻ spleen cells were transferred to WT recipients, and 2 d later, the mice were treated with integrin-neutralizing antibodies for 18 h during continuous administration of saline or clenbuterol (40 mg/kg/day). Fractions of transferred CD4⁺ T cells (CD4⁺; A) and B cells (CD19⁺; B) remaining in mesenteric LNs were determined as ratios relative to those in saline-treated mice that did not undergo integrin blockade. Fold increases of remaining fractions by clenbuterol over saline treatment are shown in the right panels. (C and D) WT mice were treated s.c. with saline or AMD3100 (5 mg/kg), and 2 h later, CD4⁺ T cells (CD4⁺) and B cells (CD19⁺) in blood (C) and lymph (D) were quantified. (E–G) WT mice were treated with integrin-neutralizing antibodies for 18 h during continuous administration of saline, AMD3100 (40 mg/kg/day, E) or combination of AMD3100 and clenbuterol (40 mg/kg/day of each, F and G). Fractions of CD4⁺ T cells (CD4⁺, E and F) and B cells (CD19⁺IgD⁺CD95⁻, E and G) remaining in mesenteric LNs were determined as ratios relative to those in saline-treated mice that did not undergo integrin blockade. Fold increases of remaining fractions by clenbuterol treatment are shown in the right panels (F and G). Dashed lines show the levels of saline-treated control as a ratio of 1 (A, B, F, and G). Data are pooled from two experiments. Each symbol represents an individual mouse and bars indicate means. Sal, saline; Clen, clenbuterol. *, *P < 0.05; **, *P < 0.01; ***, *P < 0.001; ****, *P < 0.0001; ns, not significant. P-values were obtained by one-way ANOVA with Bonferroni’s post-test (left panels of A, B, F, and G) or unpaired Student’s t test (C–E and right panels of A, B, F, and G).

Signals through β₂ARs suppress T cell–mediated inflammation

Analogous to the action of FTY720, we hypothesized that β₂AR–mediated control of lymphocyte egress might affect the pathogenesis of inflammatory diseases. Indeed, previous studies showed that treatment with β₂AR agonists suppressed experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis (Chelmicka-Schorr et al., 1989; Wiegmann et al., 1995), although the mechanisms of their action were not addressed. To test the role of β₂ARs in T cell–mediated inflammation, we used mouse EAE and delayed-type
hypotheses (DTH) responses in the skin. We treated mice with clenbuterol in the course of EAE induced by immunization with myelin oligodendrocyte glycoprotein (MOG) in adjuvant. To target the trafficking of MOG-primed encephalitogenic T cells from LNs to the central nervous system without affecting their generation, the β2AR agonist was administered for 3 consecutive days at the onset of disease. Consistent with the previous studies, treatment inhibited the progression of paralytic symptoms characteristic of EAE (Fig. 6 A). Conversely, β2AR-deficient mice developed more severe symptoms compared with β2AR–sufficient mice (Fig. 6 B), suggesting that physiological levels of inputs through β2ARs are involved in EAE pathology. The disease induced in β2AR–deficient mice was not affected by clenbuterol treatment (Fig. 6 C), indicating β2AR specificity of clenbuterol in an inflammatory condition.

To induce skin DTH responses, we immunized mice with OVA in adjuvant, and 7 d later, ear skin was challenged with an intradermal injection of OVA that induced inflammation and ear swelling peaking ~48 h later. Consistent with results in the EAE model, continuous administration of the β2AR agonist before OVA challenge in the ear attenuated the DTH responses (Fig. 6 D). Additionally, β2AR–deficient mice exhibited an enhanced OVA-induced DTH response (Fig. 6 E) and unresponsiveness to clenbuterol treatment (Fig. 6 F). Despite increased inflammatory responses, β2AR deficiency did not affect the generation of disease-related CD4+ T helper cells producing IL-17 (Th17) or IFN-γ (Th1) in the draining inguinal LNs of MOG- or OVA-immunized mice (Fig. 6, G and H; and Fig. S6). Collectively, these data establish that signals through β2ARs act to attenuate T cell–mediated inflammation. Although stimulation of β2ARs had some impacts on the differentiation of T helper cells in cultures (Sanders et al., 2012), our results obtained from β2AR-deficient mice suggest that physiological inputs through β2ARs might play a minimal role in antigen-induced T cell differentiation in vivo.

**Signals through β2ARs inhibit LN egress of antigen-primed T cells**

As an approach to reveal the role of β2AR–mediated signals in T cell dynamics in an inflammatory condition, we used the skin DTH model in which trafficking of adoptively transferred antigen-specific T cells could be tracked. We first tested the effect of β2AR stimulation on T cell recruitment into the ear in the initial phase of inflammation. Naive OT-II T cells expressing OVA-specific TCRs were transferred into recipient mice and then the mice were immunized with OVA in adjuvant. 7 d later, the immunized mice were subjected to continuous administration of clenbuterol through osmotic pumps, followed by OVA challenge in the ear. The number of OT-II T cells recruited into the inflamed ear 12 h after challenge was substantially reduced, but not completely blocked, by treatment with the β2AR agonist (Fig. 7 A and Fig. S7 A). Concomitantly, reduction of OT-II T cells in blood and lymph was observed (Fig. 7 B and Fig. S7 B), suggesting that β2AR–mediated inhibition of LN egress of OT-II T cells might be the cause of their impaired recruitment into the ear.

To assess the egress rate of antigen-primed OT-II T cells from LNs, we performed entry blockade with integrin-neutralizing antibodies upon β2AR stimulation 7 d after immunization. At this time, all transferred OT-II T cells in the draining inguinal LNs exhibited an activated phenotype demonstrated by the up-regulation of CD44 (Fig. S7 C). Administration of clenbuterol dose-dependently enhanced the retention of CD44hiCD62Lhi central memory OT-II T cells, but not CD44hiCD62Lhi effector memory OT-II T cells, in the inguinal LNs (Fig. 7 C and Fig. S7C). Consistent with the established phenotypes of central memory T cells (Sallusto et al., 2004), CD44hiCD62Lhi OT-II T cells had higher expression of CCR7, whereas CXCR4 was more abundantly expressed in CD44hiCD62Lhi OT-II T cells (Fig. 7 D). The expression of β2AR transcripts was higher in central memory OT-II T cells than in effector memory cells (Fig. 7 E). The expression pattern of these receptors suggests that higher expression of CCR7 and β2ARs might contribute to the preferential retention of central memory T cells. These findings suggest that stimulation of β2ARs in the course of T cell–mediated inflammation selectively inhibits egress of central memory T cells from LNs.

To examine the effect of β2AR deficiency on the dynamics of antigen-primed T cells, we transferred β2AR–sufficient or –deficient OT-II T cells (CD45.2+) together with WT OT-II T cells (CD45.1+CD45.2+) as an internal control and immunized the recipient mice (CD45.1+) with OVA in adjuvant. Consistent with the results obtained from MOG- or OVA-immunized β2AR–deficient mice, we found that generation and differentiation of antigen-primed OT-II T cells were not affected by β2AR deficiency (Fig. 7 F and G; and Fig. S7, D and E). Nevertheless, recruitment of β2AR–deficient OT-II T cells to the inflamed ear was enhanced compared with β2AR–sufficient cells (Fig. 7 H and Fig. S7 F). Additionally, β2AR–deficient OT-II T cells were enriched in blood and lymph of the immunized mice (Fig. 7 I and Fig. S7 G), suggesting that LN egress of antigen-primed OT-II T cells is promoted by β2AR deficiency. Indeed, β2AR deficiency resulted in more rapid egress of CD44hiCD62Lhi central memory OT-II T cells from the draining inguinal LNs relative to co-transferred WT control cells (Fig. 7 J). As expected from the low expression of β2ARs in CD44hiCD62Lhi effector memory OT-II T cells (Fig. 7 E), their LN egress was not affected by β2AR deficiency (Fig. 7 J). Because the expression of CCR7 and CXCR4 was comparable between β2AR-deficient and WT control OT-II T cells (unpublished data), the impaired LN retention was not attributable to reduced expression of these chemokine receptors. These findings indicate that physiological inputs through β2ARs expressed on antigen-primed T cells control their dynamics in an inflammatory condition. However, we cannot exclude the contribution of β2ARs expressed on other cell types, including endothelial cells, in which β2AR–mediated signals are involved in the expression of adhesion molecules (Scheiermann et al., 2012).
Figure 6. β2AR-mediated signals attenuate T cell–mediated inflammation. (A) WT mice were immunized with MOG35-55 peptide and scored for clinical signs of EAE. Saline (n = 7) or clenbuterol (0.4 mg/kg/day, n = 7) was injected i.v. for 3 consecutive days at the disease onset. (B) EAE was induced in Adrb2+/− (n = 10) and Adrb2−/− (n = 12) mice. (C) Adrb2+/− and Adrb2−/− mice were treated with saline or clenbuterol (0.4 mg/kg/day) at the onset of EAE (at least six mice per group). Clinical scores are shown as mean + SEM. The days of drug treatment are indicated by arrows (A and C). (D) 7 d after immunization with OVA protein, WT mice received continuous 3-d administration of saline (n = 5) or clenbuterol (40 mg/kg/day, n = 5), and were challenged with OVA in the ear. DTH responses were assessed by ear swelling at the indicated times. (E) OVA-induced DTH responses were measured in Adrb2+/− (n = 8) and Adrb2−/− (n = 8) mice as in D. (F) DTH was induced in Adrb2+/− and Adrb2−/− mice during administration of saline or clenbuterol (40 mg/kg/day, five mice per group) and measured as in D. Ear swelling is shown as mean + SEM [D–F]. (G and H) Frequencies (top) and numbers (bottom) of Th17 (CD3+CD4+IL-17+IFN-γ−) and/or Th1 (CD3+CD4+IFN-γ+IL-17−) cells were analyzed in the draining inguinal LNs of Adrb2+/− (+/−) and Adrb2−/− (−/−) mice 14 d after immunization with MOG (G) or 7 d after immunization with OVA (H). The dashed lines show the background levels in the inguinal LNs of unimmunized WT mice. Each symbol represents an individual mouse and bars indicate means (G and H). Data are representative of four (A) or two (C, D, and F) experiments, or pooled from two experiments (B, E, G, and H). Sal, saline; Clen, clenbuterol. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ns, not significant. P-values were obtained by Mann–Whitney U test (A–G) or unpaired Student’s t test (D–H).
Figure 7. β₂AR-mediated signals inhibit LN egress of antigen-primed T cells. (A–C) WT OT-II T cells (CD45.2+) were transferred into WT mice (CD45.1+), and the recipients were immunized with OVA. 7 d later, the mice were subjected to continuous administration of saline or clenbuterol (40 mg/kg/day). (A) OT-II T cells recruited to the ear were quantified by flow cytometry 12 h after OVA challenge. The graph shows numbers of OT-II T cells per 10⁵ cells in saline-injected or OVA-challenged ears. (B) Numbers of OT-II T cells in blood and lymph of immunized mice were enumerated after 12-h clenbuterol treatment. (C) The immunized mice were treated with integrin-neutralizing antibodies for 20 h during treatment with saline or the indicated doses of clenbuterol. Fractions of central memory (CD44hiCD62Lhi) and effector memory (CD44hiCD62Llo) OT-II T cells remaining in the draining inguinal LNs were determined as ratios relative to those of immunized mice treated with saline but not integrin-neutralizing antibodies. Data are shown as mean ± SEM for at least three mice. (D) Surface expression of CCR7 and CXCR4 on CD44hiCD62Llo and CD44hiCD62Lhi OT-II T cells in the inguinal LNs. (E) Adrb2 mRNA transcript abundance was measured by quantitative RT-PCR in naive (CD44loCD62Lhi), CD44hiCD62Lhi, and CD44hiCD62Llo OT-II T cells, and quantified relative to the abundance of Gapdh. Data are shown as mean ± SD of triplicates. (F–J) CD45.1+ WT mice received a mixture of CD45.1+CD45.2+ and CD45.2+ OT-II T cells (Adrb2+/− mix) or mixture of CD45.1+CD45.2− WT and CD45.2− Adrb2+/− OT-II T cells (Adrb2+/− mix), and were immunized with OVA. 7 d later, the mice were subjected to analysis. Normalized ratios of CD45.2+ cells relative to CD45.1+ CD45.2− cells were obtained by...
DISCUSSION
This study demonstrates that activation of \( \beta_2 \)ARs in lymphocytes enhances the responsiveness of retention-promoting chemokine receptors to inhibit lymphocyte egress from LNs. Under physiological conditions, the inputs to \( \beta_2 \)ARs are provided at least in part by adrenergic nerves. Thus, this study has revealed a cell-intrinsic mechanism by which the nervous system controls lymphocyte recirculation through LNs. This marks a sharp contrast to the role of adrenergic nerves in controlling BM egress of hematopoietic progenitor cells and tissue recruitment of myeloid cells, in which adrenergic inputs affect the expression of adhesion molecules and chemokines in nonhematopoietic compartments of the microenvironments (Méndez-Ferrer et al., 2008; Scheiermann et al., 2012).

However, the sites of interplay between adrenergic nerves and lymphocytes remain to be determined. A previous study demonstrated that substantial levels of noradrenaline were detectable in T cell zones and interfollicular areas but not in B cell follicles in LNs (Felten et al., 1985). Thus, T cells might be constantly exposed to noradrenaline in LNs, whereas B cells might receive the inputs only when they travel to LN exit portals called cortical sinuses, which are located at the interface between T cell zones and B cell follicles (Grigorova et al., 2009; Sinha et al., 2009; Pham et al., 2010). Continuous exposure to noradrenaline would promote desensitization of \( \beta_2 \)ARs in T cells, which might be an additional cause for the lower responsiveness of T cells to augmentation or depletion of \( \beta_2 \)AR-mediated inputs compared with B cells. To understand how adrenergic nerves control lymphocyte behaviors, it will be important to visualize interactions between adrenergic nerves and lymphocytes using intravital microscopy.

Additionally, the physiological significance of \( \beta_2 \)AR-mediated control of lymphocyte trafficking is currently unclear. Studies on circadian regulation of immunity suggested that the innate immune system prepares for higher risk of infection during the active phase, when the activity of adrenergic nerves is high, by up-regulating pathogen sensors (Silver et al., 2012) and recruiting myeloid cells into peripheral tissues (Scheiermann et al., 2012; Nguyen et al., 2013). Our data suggest that \( \beta_2 \)AR-mediated signals sequester lymphocytes into lymphoid organs from the circulation during the period of high adrenergic activity. Considering that lymphocytes specific for a given antigen are very rare (20–200 cells/mouse) and the population size dictates magnitude of the response (Moon et al., 2007), sequestration of lymphocytes in lymphoid organs during the active phase may increase the chance of antigen encounter and potentiate adaptive immune responses.

Although stimulation of \( \beta_2 \)ARs increased the responsiveness of both CCR7 and CXCR4 in vitro (Fig. 4, A and D), the enhanced LN retention of B and T cells was dependent on distinct chemokine receptors: CCR7 in B cells and CXCR4 in T cells (Fig. 5, A, B, F, and G). In Transwell migration assays, T cells exhibit higher chemotactic responses to the CCR7 ligand than to the CXCR4 ligand, whereas B cells show an opposite trend of responses (unpublished data). Therefore, the effect of \( \beta_2 \)AR stimulation on LN retention may be masked by the strong signals evoked by CCR7 in T cells or CXCR4 in B cells, and \( \beta_2 \)AR-promoted LN retention may rather reflect signal enhancement of CXCR4 in T cells or CCR7 in B cells. This might explain the observation that retention-promoting effects of \( \beta_2 \)AR stimulation were abolished only when functions of the less-dominant chemokine receptors were blocked.

CCR7 deficiency ( Förster et al., 1999 ) and CXCR4 deficiency in the absence of CCR7-mediated signals (Okada et al., 2002) resulted in significant reduction of lymphocyte homing to LNs, establishing the contribution of CCR7 and CXCR4 to lymphocyte entry to LNs. However, lymphocyte homing was unaffected by agonist activation (Fig. 2 C) or genetic ablation (Fig. 3 C) of \( \beta_2 \)AR-mediated signals. During lymphocyte entry from blood to LNs, signals through chemokine receptors induce activation of \( \alpha_4 \) and \( \alpha_L \) integrins, which is essential for lymphocyte to firmly adhere to the lumen of high endothelial venules (von Andrian and Mempel, 2003). However, lymphocyte egress from LNs does not require the integrins (Lo et al., 2005) and the rate of egress appears to depend on the relative strength of egress-promoting signals and retention-promoting signals (Pham et al., 2008). We speculate that basal levels of CCR7- or CXCR4-mediated signals might be sufficient for optimal integrin activation, allowing efficient lymphocyte homing irrespective of inputs through \( \beta_2 \)ARs. This might explain the fact that signal enhancement of CCR7 and CXCR4 by \( \beta_2 \)AR activation is selectively reflected in lymphocyte egress from LNs.

Heterodimerization of GPCRs is an emerging concept that accounts for their functional and pharmacological properties (Milligan, 2009; Prezeau et al., 2010). Our data suggest that \( \beta_2 \)ARs might selectively form heteromeric complexes with CCR7 or CXCR4, although a definitive demonstration of their interactions in primary lymphocytes has not been achieved. Because \( \beta_2 \)AR stimulation preferentially enhances chemotaxis through distinct chemokine receptors: CCR7 and CXCR4 (Fig. 4, A and D), the physical association could be key to functional crosstalk between \( \beta_2 \)ARs and chemokine receptors. Heteromerization...
might have allostatic effects on the conformation of chemo-
kine receptors that favor ligand binding or signal transduc-
tion, and help converge and synergize their signaling pathways
with those of β2ARs. These possibilities will be tested in fu-
ture studies to reveal the molecular mechanism of the cross
talk between the two classes of GPCRs.

We demonstrated that signals through β2ARs inhibited
LN egress of antigen-primed T cells, reduced their numbers in
circulation, and impaired their recruitment to inflamed per-
ipheral tissues (Fig. 7). Although it is possible that these β2AR-
mediated effects on lymphocyte dynamics might contribute
to the attenuated T cell–induced inflammation (Fig. 6), the
direct causal connection has not been established. Previous stud-
ies showed that stimulation of β2ARs in dendritic cells re-
duced their capacities for antigen presentation and production
of proinflammatory cytokines, including IL-6, IL-12, and IL-23
(Grebe et al., 2009; Hu et al., 2012; Hervé et al., 2013; Nijhuis
et al., 2014). Therefore, the alteration of dendritic cell func-
tions might impair reactivation of central memory T cells at
sites of inflammation. We speculate that combination of these
β2AR–mediated effects on immune functions might contribut-
to the suppression of inflammatory responses.

A recent study showed that inputs from adrenergic nerves
induced production of a chemokine CCL20 from blood ves-
sels to promote invasion of encephalitogenic T cells into the
central nervous system during the onset of EAE (Arima et al.,
2012). This effect of adrenergic stimulation appeared to be
mediated by β2ARs because treatment with a selective β2AR
antagonist diminished chemokine production by blood ves-
sels and ameliorated disease. Combined with our results, these
findings indicate that stimulation of distinct adrenergic recep-
tors β1 and β2 might produce opposite outcomes in EAE.
Thus, it would be worth exploring the therapeutic potential
of β2AR stimulation, possibly in combination with β2AR
blockade, in T cell–mediated inflammatory diseases.

MATERIALS AND METHODS

Mice. C57BL/6 (B6) mice were obtained from Clea, CD45.1+ and Cd7−/−
(CD7tm1Rfor, MGI: 2180679) mice on a B6 background were purchased from
The Jackson Laboratory. Adhb−/− mice on a B6 background were generated
as described previously (Hanyu et al., 2012). OT-I/II TCR transgenic mice on
a B6 background were provided by W.R. Heath (The University of Melbourne,
Parkville, Victoria, Australia; Barnden et al., 1998) and crossed to Adhb−/− or
CD45.1+ WT mice to generate Adhb−/− or CD45.1−CD45.2+ WT OT-II mice,
respectively. 8–12 wk-old B6 mice were used for experiments. BM chimeras
were generated by irradiating recipient mice with a single dose of 8 Gy,
followed by i.v. transfer of BM cells. Chimeras were analyzed at least 8 wk
after irradiation. Mice were housed in a specific pathogen–free facility, and all
experiments were performed in accordance with protocols approved by the
Osaka University Animal Care Committee.

Flow cytometry and cell sorting. Lymphoid tissues were disrupted by
passage through a 40-μm cell strainer (BD). For blood samples, red blood cells
were lysed with ammonium chloride potassium (ACK) buffer. Single cells were
stained with the following fluorochrome-labeled antibodies (BioLegend):
CD3ε (145-2C11), CD4 (RM4-5), CD8α (53-6.7), CD19 (6D5), CD11b
(M1/70), CD21/CD35 (7E9), CD23 (B3B4), CD44 (IM7), CD45 (30-F11),
CD45.1 (A20), CD45.2 (104), CD62L (MEL-14), CD93 (AA4.1), CCR7
(4B12), IgD (11-26c.2a), ly 6G (1A8), TCRβ (H57-597), and TCR Vα2
(B20.1). Antibodies against CD95 (Jo2) and CXCR4 (2B11) were purchased
from BDA For analysis of intracellular cytokines, cells were fixed and permea-
bilized with Cytofix/Cytoperm solution (BD) after surface staining and stained
with antibodies against IFN-γ (XMG1.2) and IL-17A (TC11-18H10.1; both from
BioLegend). Data were acquired on a FACSVersus cytometer (BD) and
analyzed with FlowJo software (Tree Star). Cell sorting was performed on a
FacsAria II (BD) to isolate the following populations: follicular B cells
(CD19+CD93−CD23hiCD21lo), CD4+ T cells (CD4+CD44hiCD62Llo), and
CD8+ T cells (CD8+CD44loCD62Lhi) from the spleen; naive OT-II T cells
(CD4+Vα2−CD4+CD62Lhi) from the spleen of OT-II mice; and OT-II T
cells (CD4+Vα2+CD4−CD62Llo) with a central memory (CD4+CD62Lhi)
or effector memory (CD44hiCD62Llo) phenotype from the draining LNs of
OVA-immunized mice that had received OT-II T cells.

Constructs and retroviral transduction. The MSCV2.2 retroviral vec-
tors expressing Flag-tagged mouse CCR7, CXCR4, or S1PR1, upstream of
an internal ribosomal entry site (IRES) and a cytoplasmic domain–truncated
human CD4, were provided by J.G. Cyster (University of California, San
Francisco, San Francisco, CA; Lo et al., 2005). Mouse CXCR4 was also
cloned into this vector. Mouse β2AR was cloned from splenic cDNA and
inserted downstream of an IRES. Cultures of Phoenix-E packaging cell line were
transfected with these constructs, supernatants containing retrovirus were collected and 2PK-3 cells (ATCC) were transduced as described (Lo et al., 2005).

Immunoprecipitation and pull-down assay. 2PK-3 cells were stably
transduced with Myc-tagged β2AR and Flag-tagged CCR7, CXCR4,
CXCR5, or S1PR1. The transduced 2PK-3 cells (8 × 10⁶ cells) were washed
with PBS and lysed in 0.8 ml lysis buffer (0.875% Brij 97, 0.125% Nonidet
P-40, 150 mM NaCl, and 10 mM Tris-HCl, pH 7.2) containing protease
inhibitors (Nacalai Tesque). Supernatants were preclarified before incubation
with Flag M2 beads (Sigma–Aldrich) for 12 h at 4°C. Beads were washed
with lysis buffer containing 0.5 M NaCl. Samples were eluted and reduced
before separation in NuPAGE 4–12% Bis-Tris gels (Life Technologies) and
transferred to Immobilon-P2 membranes (EMD Millipore). Membranes
were blocked with 4% skim milk in TBS containing 0.1% Tween 20 and
blotted with mouse monoclonal anti-Flag (M2; Sigma–Aldrich) or rabbit
polyclonal anti-Myc (Cell Signaling Technology), followed by goat anti–mouse
IgG or donkey anti–rabbit IgG conjugated with horseradish peroxidase (HRP;
both from Jackson ImmunoResearch Laboratories). Activation of Rac1 was
analyzed using an assay kit from Millipore. In brief, 10⁶ B or T cells, isolated
from mouse spleens by negative selection using AutoMACS (Miltenyi Biotec),
were stimulated with chemoattractants in the presence or absence of 10 μM
clenbuterol (Sigma–Aldrich). Cell extracts were incubated with a Rac-binding
domain of PKAI bound to agarose beads for 1 h at 4°C. Reduced eluates
were resolved in NuPAGE 10% Bis-Tris gels (Life Technologies) and
blotted with mouse monoclonal anti-Rac1 (23A8), followed by HRP-conjugated
goat anti–mouse IgG (Jackson ImmunoResearch Laboratories). Images were
acquired with a luminescent image analyzer (ImageQuant LAS4000 mini;
GE Healthcare), and band density was analyzed with ImageJ software (National
Institutes of Health).

Immunofluorescence. For single-cell analysis of 2PK-3 cells expressing
Myc-tagged β2ARs together with Flag-tagged CCR7, CXCR4, or S1PR1,
cells were stained with Alexa Fluor 647–conjugated anti-Myc (9B11; Cell
Signaling Technology) and biotin-labeled antibodies against CCR7 (4B12)
or CXCR4 (2B11), followed by PE-labeled streptavidin (BioLegend). S1PR1
was detected with a purified rat antibody (713412; R&D Systems), followed by
biotin-labeled anti–rat IgG (Jackson ImmunoResearch Laboratories) and
PE-labeled streptavidin (Arnon et al., 2011). Labeled cells were seeded onto
slides and fixed with 4% paraformaldehyde. Slides were mounted with Fluor-
Save Reagent (EMD Millipore). Images were acquired at room temperature
with Fluoview FV10-ASW version 3.00 using a Fluoview FV1000 inverted
confocal microscope (Olympus), equipped with plan apochromat UPLSAPO
Quantitative RT-PCR. Total RNA was extracted with a Nucleospin RNA kit (Macherey-Nagel) from FACSSorted cells. Complementary DNA was synthesized with a ReverTra Ace qPCR RT kit (Toyobo). Quantitative PCR was performed with SYBR Premix Ex Taq II on a Thermal Cycler Dice Real Time System II (Takara). The following primers were used to detect Adh2 transcripts: 5′GTCACGGTCACCCACAGA−3′ (forward) and 5′CCC-GGAAATTGACAAAGCATTCT−3′ (reverse).

Transwell migration assay. Spleen cells from WT and Adh2−/− mice were treated with ACK buffer to lyse red blood cells, washed five times, and incubated for 30 min at 37°C in RPMI containing 0.5% fatty acid-free BSA (EMD Millipore). Cells were put into the upper chambers of Transwells (Costar) and tested for transmigration across filters with 5-µm pores for 3 h at 37°C in response to chemoattractants in the lower chambers: 100 ng/ml CCL21 (R&D Systems), 10 ng/ml CXCL12 (R&D Systems), 300 ng/ml CXCL13 (R&D Systems), or 100 nM S1P (Sigma−Aldrich). Clenbuterol was added to the lower chambers with the chemoattractants. CD4+ or CD8+ T cells and B cells that migrated to the lower chambers were enumerated by flow cytometry and chemotactic responses were determined as percentages of their numbers relative to those of input cells.

Collection of blood and lymph. After mice were euthanized with CO2, lymph was drawn from the cysterna chyli using a fine microcapillary pipette (Malobadian et al., 2004), and then blood was collected from the inferior vena cava using a syringe with a 26-gauge needle. To determine the cell concentration in lymph, cell numbers counted by flow cytometry were divided by the volume of collected lymph. Except for the time course analysis of lymphocyte numbers after treatment with β2AR agonists, blood and lymph were collected between 2 and 4 h after the onset of light to avoid circadian variation of lymphocyte counts.

Lymphocyte egress from LNs. Lymphocyte entry to LNs was blocked by i.v. injection of neutralizing antibodies against integrin αL (M17/4) and α4 (PS/2, both from Bio X Cell) at 100 µg each per mouse and, 14−22 h later, lymphocytes remaining in the LNs were enumerated (Lo et al., 2005). Mini osmotic pumps (2001D, Alzet) containing saline, AMD3100 (40 mg/kg/d; Sigma−Aldrich) and/or various doses of clenbuterol or salbutamol were subcutaneously implanted s.c. in the back of mice to continuously administer the drugs for 1 week. Flow cytometry was performed on day 14 and disrupted by passage through a 70-µm cell strainer (BD) and subjected to flow cytometry for detection of central memory and effector memory OTII-T cells. For analysis of T cell recruitment to inflammatory sites, ears were injected i.d. with saline or 20 µg OVA 7 d after immunization, and collected 12 h later. The dorsal half of the ear was treated with 1 U/ml Disperse I (Roche) in RPMI containing 1% FBS for 1 h at 37°C to remove the dermis. The dermis was cut into pieces and digested with 0.3 mg/ml Liberase TL (Roche) and 0.085% DNase I (Roche) in RPMI containing 1% FBS for 1.5 h at 37°C. The tissue was disrupted by passage through a 70-µm cell strainer (BD) and subjected to flow cytometry for detection of OT-II T cells.

DTH. Mice were immunized s.c. with 100 µg OVA emulsified in CFA at the base of the tail. 7 d after immunization, ears were injected i.d. with saline or 20 µg OVA. Mini osmotic pumps (1003D, Alzet) containing saline or clenbuterol (40 mg/kg/day) were implanted 1 h before DTH and left for 3 d. Thickness of the ear was measured with a spring-loaded caliper (Mitutoyo). Net ear swelling was calculated by subtracting the increased thickness of the saline-injected ear from that of the OVA-injected ear. For analysis of effector T cell generation, single cells prepared from the inguinal LNs 7 d after immunization were stimulated and stained for intracellular cytokines as described above.

Analysis of T cell trafficking in DTH. To analyze the dynamics of OT-II T cells in the DTH model, 2 × 105 CD45.2+ WT OT-II T cells or mixtures of CD45.2+ Adh2−/− or Adh2−/− plus CD45.1+ CD45.2+ WT OT-II T cells (2 × 105 cells of each) were transferred i.v. into CD45.1+ mice and then the mice were immunized with 100 µg OVA in CFA the next day. In some experiments, clenbuterol was administered through mini osmotic pumps (2001D) 7 d after immunization. For analysis of LN egress, entry blockade was performed 7 d after immunization. The draining inguinal LNs were collected after the indicated time and subjected to flow cytometry for detection of central memory and effector memory OTII-T cells. For analysis of T cell recruitment to inflammatory sites, ears were injected i.d. with saline or 20 µg OVA 7 d after immunization, and collected 12 h later. The dorsal half of the ear was treated with 1 U/ml Disperse I (Roche) in RPMI containing 1% FBS for 1 h at 37°C to remove the dermis. The dermis was cut into pieces and digested with 0.3 mg/ml Liberase TL (Roche) and 0.085% DNase I (Roche) in RPMI containing 1% FBS for 1.5 h at 37°C. The tissue was disrupted by passage through a 70-µm cell strainer (BD) and subjected to flow cytometry for detection of OT-II T cells.

Statistical analysis. GraphPad Prism (GraphPad Software) was used for all statistical analyses. For comparison of two nonparametric datasets, Mann−Whitney U test was used. Means of two groups were compared with unpaired Student’s t test. For multigroup comparisons, we applied one-way ANOVA with post hoc testing using Bonferroni’s multiple comparison test. The ED50 values were obtained from nonlinear regression analysis of the curves.

Online supplemental material. Figs. S1−S7 show representative flow cytometry plots and gating strategies for the data shown in Figs. 1−7, respectively. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20141132/DC1.

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