Control of lymphocyte egress from lymph nodes through β_2-adrenergic receptors

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Lymphocyte recirculation through secondary lymphoid organs is essential for immunosurveillance and lymphocyte effector functions. Here, we show that signals through β_2-adrenergic receptors (β_2ARs) expressed on lymphocytes are involved in the control of lymphocyte dynamics by altering the responsiveness of chemoattractant receptors. Agonist stimulation of lymphocyte β_2ARs inhibited egress of lymphocytes from lymph nodes (LNs) and rapidly produced lymphopenia in mice. Physiological inputs from adrenergic nerves contributed to retention of lymphocytes within LNs and homeostasis of their distribution among lymphoid tissues. β_2ARs physically interacted with CCR7 and CXCR4, chemokine receptors promoting lymphocyte retention in LNs. Activation of β_2ARs enhanced retention-promoting signals through CCR7 and CXCR4, and consequently inhibited lymphocyte egress from LNs. In models of T cell–mediated inflammatory diseases, β_2AR-mediated signals inhibited LN egress of antigen-primed T cells and reduced their recruitment into peripheral tissues. Thus, this study reveals a novel mechanism for controlling lymphocyte trafficking and provides additional insights into immune regulation by the nervous system.

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Abbreviations used: 6-OHDA, 6-hydroxydopamine; β_2AR, β_2-adrenergic receptor; DTH, delayed-type hypersensitivity; EAE, experimental autoimmune encephalomyelitis; GPCR, G protein–coupled receptor; MOG, myelin oligodendrocyte glycoprotein; S1PR1, sphingosine-1-phosphate receptor-1.

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 chemoattractant 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 β-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 blood stream 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

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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 β_2-adrenergic receptors (β_2ARs), 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 β_2ARs sequesters antigen-primed T cells in LNs and prevents their migration to inflamed tissues, suggesting a mechanism for β_2AR-mediated suppression of inflammatory responses.

RESULTS

Stimulation of β_2ARs causes lymphopenia by a cell-intrinsic mechanism

Because β_2ARs are predominantly expressed in lymphocytes compared with other subtypes of adrenergic receptors (Sanders, 2012), we treated mice with selective β_2AR agonists, clenbuterol, or salbutamol, to mimic activation of adrenergic nerves and test the possible role of β_2ARs in lymphocyte dynamics. Administration of a single dose of either β_2AR 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 β_2AR agonists (Smith, 1998), clenbuterol was more potent than salbutamol. The ED_{50} 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 β_2AR 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 β_2AR stimulation was prominent in B cells compared with T cells, which was consistent with the predominant expression of transcripts for β_2ARs in B cells (Fig. 1 E and Fig. S1, C and D). Flow cytometric analysis of the surface expression of β_2ARs 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 β_2AR-deficient mice (Fig. 1, F and G; and not depicted), confirming that clenbuterol specifically acts on β_2ARs in this experimental setting. To determine whether the action of the β_2AR agonist was mediated by β_2ARs expressed on the radiosensitive hematopoietic compartment or radioresistant nonhematopoietic compartment, we administered clenbuterol to BM chimeras generated using β_2AR-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 β_2AR-deficient BM cells (Fig. 1, H and I). In contrast, B cell numbers in β_2AR-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 β_2AR agonist largely depends on β_2ARs expressed on hematopoietic cells although the involvement of nonhematopoietic β_2ARs cannot be excluded.

Lymphocyte egress from LNs is inhibited by β_2AR 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 β_2ARs 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 β_2AR 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 β_2AR 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 β_2AR 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 β_2AR-deficient BM cells, LN retention of B and T cells was not promoted by clenbuterol treatment (Fig. 2 D and not depicted), whereas β_2AR stimulation resulted in enhanced lymphocyte retention in the LNs of β_2AR-deficient mice reconstituted with WT BM cells (Fig. 2 E and not depicted). These observations indicate that stimulation of β_2ARs inhibits egress of lymphocytes from LNs in a cell-intrinsic manner.

Physiological inputs through β_2ARs contribute to lymphocyte retention in LNs

There was a trend for β_2AR-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 β_2ARs are involved in lymphocyte

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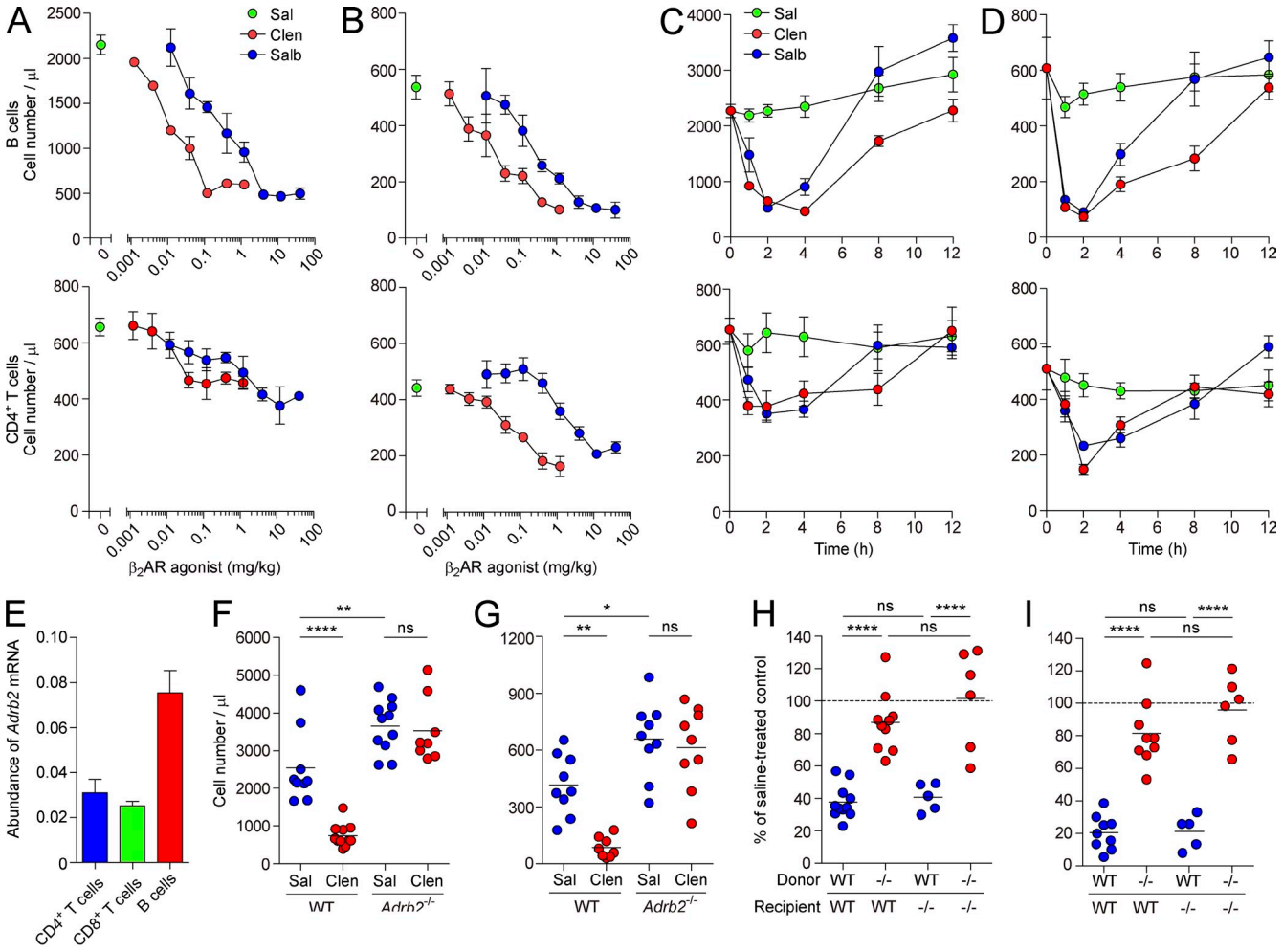

Figure 1. β_2AR stimulation induces lymphopenia in a cell-intrinsic manner. (A and B) Numbers of B cells ($CD19^+$, top) and $CD4^+$ T cells ($CD4^+$, bottom) in blood (A) and lymph (B) that were collected from WT mice 2 h after i.v. injection with the indicated doses of clenbuterol or salbutamol. Data 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 β_2AR) 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) from WT (*Adrb2*$^{+/+}$ or *Adrb2*$^{+/-}$ littermates) or *Adrb2*$^{-/-}$ mice that received i.v. injection of saline or clenbuterol (0.4 mg/kg). Data are pooled from five experiments. (H and I) BM chimeras generated using WT or *Adrb2*$^{-/-}$ (−/−) mice as donors and recipients received i.v. injection of saline or 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).

recirculation. To test the contribution of β_2ARs expressed on hematopoietic cells, we generated mixed BM chimeras using a mixture of $CD45.2^+$ β_2AR-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 β_2AR-deficient B cells over WT B cells (Fig. 3 A and Fig. S3 A). This accumulation was not observed in control chimeras generated using a mixture of congenically distinct β_2AR-sufficient and WT BM cells. These observations support that β_2AR-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 β_2ARs in T cells compared with B cells (Fig. 1 E), 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 β_2ARs in LN egress under physiological conditions, we performed short-term

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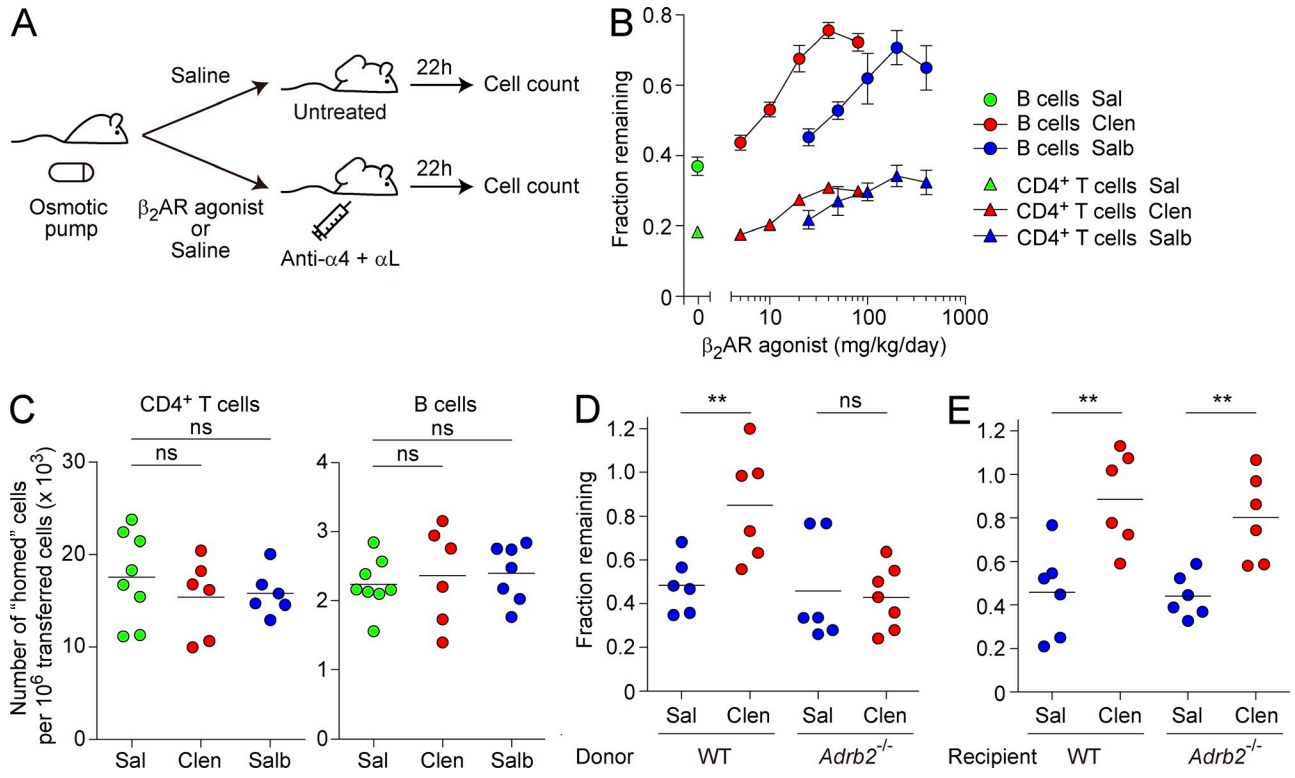

Figure 2. β_2AR stimulation inhibits lymphocyte egress from LNs. (A) Experimental design for β_2AR agonist administration and integrin blockade. During continuous administration of saline or β_2AR agonists, WT mice were treated with neutralizing antibodies against α4 and αL integrins for 22 h. Fractions 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 (CD19+IgD^{hi}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 to CD45.2+ 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 *Adrb2*$^{-/-}$ mice as donors (D) or recipients (E) were treated as in A. The remaining fraction of B cells (CD19+IgD^{hi}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; Clen, 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).

transfer experiments. β_2AR-sufficient or -deficient lymphocytes 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. β_2AR-deficient lymphocytes were retained in the mesenteric LNs to a lesser extent than β_2AR-sufficient lymphocytes (Fig. 3 B and Fig. S3 B). This result confirms that physiological inputs through β_2ARs expressed on lymphocytes inhibit their egress from LNs. Consistent with unaffected lymphocyte homing by agonist stimulation of β_2ARs (Fig. 2 C), transferred CD45.2+ β_2AR-deficient lymphocytes entered LNs as efficiently as CD45.1+ WT lymphocytes (Fig. 3 C and Fig. S3 C), further ruling out the involvement of β_2ARs 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 β_2ARs, 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 β_2ARs 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 β_2ARs might modulate

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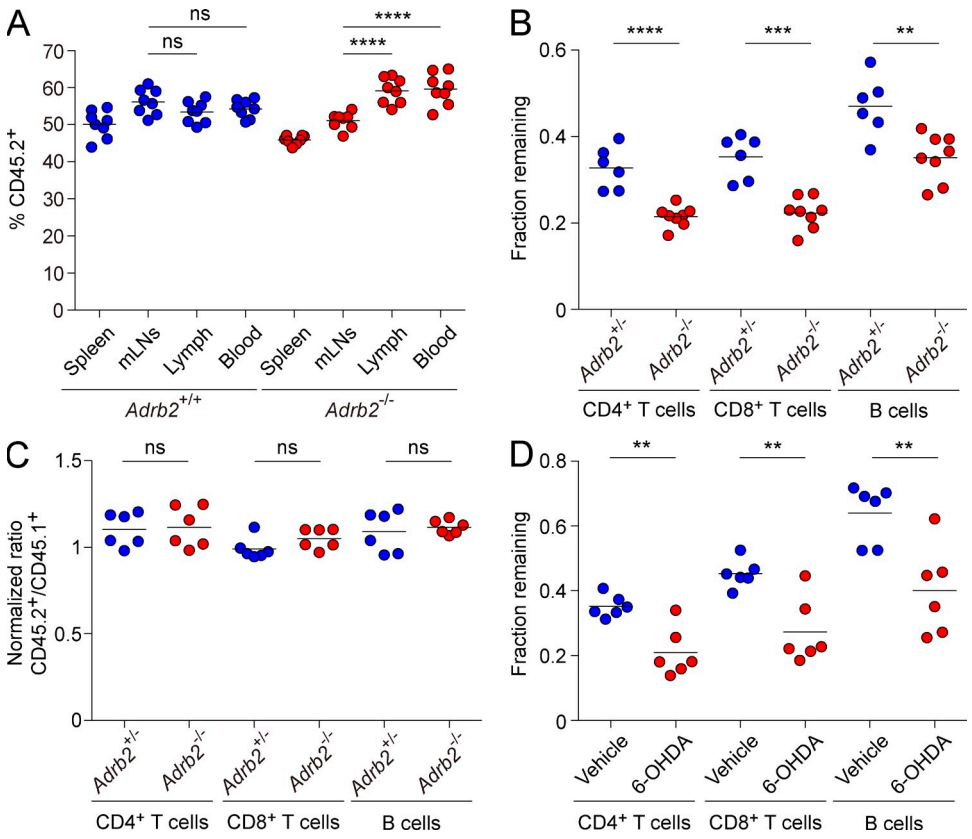

Figure 3. Physiological inputs through β_2ARs are involved in lymphocyte recirculation. (A) Flow cytometric analysis of B cell distributions in mixed BM chimeras generated with 50% *Adrb2*$^{+/+}$ or *Adrb2*$^{-/-}$ (CD45.2$^+$), plus 50% WT (CD45.1$^+$) cells. Data show percentages of CD45.2$^+$ B cells (CD19$^+$IgD^{hi}CD95$^-$) in spleen, mesenteric LNs (mLNs), lymph, and blood. (B) *Adrb2*$^{+/-}$ or *Adrb2*$^{-/-}$ spleen cells were labeled with CFSE and transferred to WT mice. 2 d later, the recipient mice were treated with integrin-neutralizing antibodies for 14 h. Fractions of transferred lymphocytes remaining in mesenteric LNs were determined as ratios relative to those in mice that did not undergo integrin blockade. (C) *Adrb2*$^{+/-}$ or *Adrb2*$^{-/-}$ (CD45.2$^+$) and WT (CD45.1$^+$) spleen cells were labeled with CFSE, co-transferred to WT (CD45.2$^+$) recipients and enumerated in mesenteric LNs 90 min after transfer. Normalized ratios of CD45.2$^+$ cells relative to CD45.1$^+$ cells were obtained by dividing observed ratios by the ratio of input populations. (D) Chemical depletion of adrenergic nerves was performed by i.p. injection of 6-OHDA into WT mice, and then mice were subjected to integrin blockade for 14 h. Fractions of lymphocytes remaining in mesenteric LNs were determined as ratios relative to those in vehicle- or 6-OHDA–treated mice that did not undergo integrin blockade. Data are representative of three experiments (A), or pooled from three (B) or two (C and D) experiments. Each symbol represents an individual mouse. Bars indicate means. **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; ns, not significant. P-values were obtained by one-way ANOVA with Bonferroni's post-test (A) or unpaired Student's *t* test (B–D).

responsiveness of these chemoattractant receptors and inhibit egress from LNs. To assess the effect of β_2AR stimulation on the signal strength of these and other lymphoid chemoattractant receptors, we analyzed the activation of a small GTPase Rac1 in a pull-down assay for GTP-bound Rac1. Stimulation with clenbuterol resulted in prolonged activation of Rac1 in response to the CCR7 ligand CCL21 and CXCR4 ligand CXCL12 in both B and T cells, whereas the effect of β_2AR stimulation was unremarkable in response to the CXCR5 ligand CXCL13 in B cells (Fig. 4 A). Treatment with S1P induced little activation of Rac1 in B and T cells, and the effect of β_2AR stimulation were not detectable (Fig. 4 A and not depicted). In β_2AR-deficient lymphocytes, the effect of clenbuterol treatment on CCR7- or CXCR4-mediated Rac1 activation were abrogated (Fig. 4 B), confirming specific action of clenbuterol on lymphocyte β_2ARs. Stimulation with the β_2AR agonist in the absence of chemoattractants did not induce activation of Rac1 in lymphocytes (Fig. 4 C). These observations suggest that stimulation of β_2ARs 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 β_2AR agonist using β_2AR-deficient lymphocytes as a reference. We used suboptimal concentrations of chemoattractants to favor the likelihood that possible chemotaxis-enhancing effects of β_2AR 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 β_2AR stimulation was detectable only in response to

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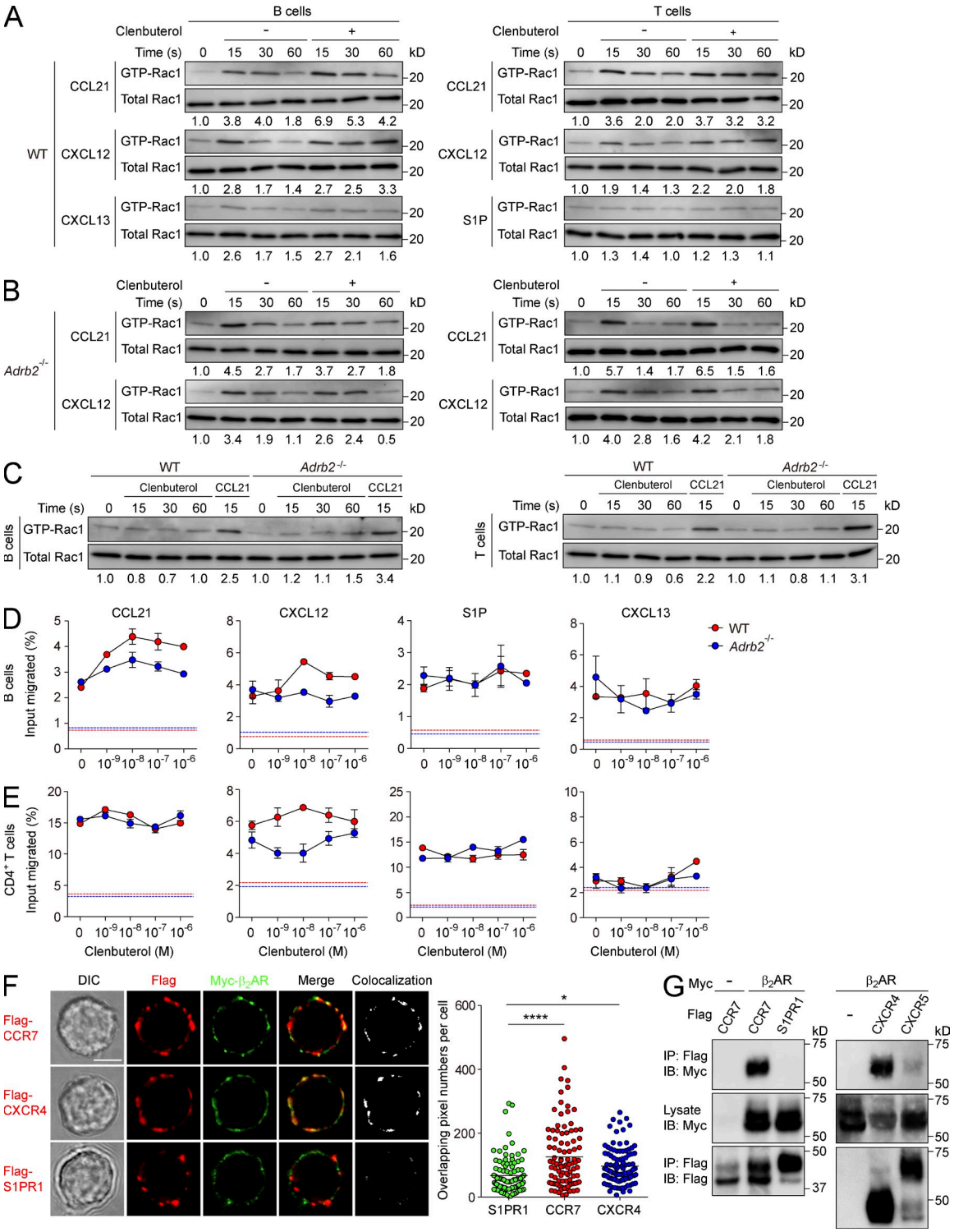

Figure 4. Functional and physical interactions of β_2ARs with chemokine receptors. (A and B) Activation of Rac1 was analyzed by Western blotting in B and T cells isolated from spleens of WT (A) or *Adrb2*$^{-/-}$ (B) mice at the indicated times after stimulation with CCL21 (2 µg/ml), CXCL12 (100 ng/ml), 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$^+$; D) and CD4$^+$ T cells (CD4$^+$; E) 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

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the CXCR4 ligand (Fig. 4 E and not depicted). The β_2AR 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 β_2AR 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 β_2ARs (unpublished data). Overall, these findings suggest that β_2AR 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 β_2ARs and CCR7 or CXCR4. Confocal microscopy for the surface localization of epitope-tagged receptors expressed in 2PK-3 B lymphoma cells demonstrated that β_2ARs co-localized more frequently with CCR7 or CXCR4 compared with S1PR1 (Fig. 4 F), suggesting that β_2ARs resides in the proximity of CCR7 or CXCR4. To examine whether the β_2ARs form complexes with CCR7 or CXCR4, we performed co-immunoprecipitation assays. Using lysates prepared from transduced 2PK-3 cells, we found that β_2ARs 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 β_2ARs 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 β_2ARs. Collectively, these findings indicate that β_2ARs are physically associated with CCR7 or CXCR4, which might contribute to the preferential enhancement of their signals by β_2AR activation.

Enhanced lymphocyte retention by β_2AR stimulation depends on CCR7 or CXCR4

The aforementioned findings suggest that stimulation of β_2ARs inhibits lymphocyte egress from LN as a consequence of enhanced lymphocyte retention through CCR7 and CXCR4. To test the involvement of CCR7 in β_2AR-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 β_2AR stimulation with clenbuterol. Treatment with the β_2AR 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 β_2AR-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 β_2AR-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 β_2AR stimulation is largely dependent on CXCR4. Thus, although we cannot exclude the involvement of other undefined retention-promoting receptors, β_2AR-mediated enhancement of LN retention of B and T cells appears to be dependent on CCR7 and CXCR4, respectively.

wells and are representative of more than three experiments for each chemoattractant. (F) Representative confocal immunofluorescence images and analysis of 2PK-3 cells expressing Myc-tagged β_2ARs (green) and Flag-tagged chemoattractant receptors (red): CCR7 (top), CXCR4 (middle), and S1PR1 (bottom). Co-localization of the signals was quantified by measurement of overlapping pixel numbers as shown in the right ($n = 95$ for S1PR1; $n = 99$ for CCR7; $n = 100$ for CXCR4). DIC, differential interference contrast. Bar, 5 µm. Data are representative of three experiments. Bars indicate means. *, $P < 0.05$; ****, $P < 0.0001$ (one-way ANOVA with Bonferroni's post-test). (G) Lysates of 2PK-3 cells expressing the indicated epitope-tagged receptors were subjected to immunoprecipitation (IP) with an anti-Flag antibody. Co-precipitated proteins were immunoblotted (IB) with an anti-Flag or anti-Myc antibody. Data are representative of three experiments.

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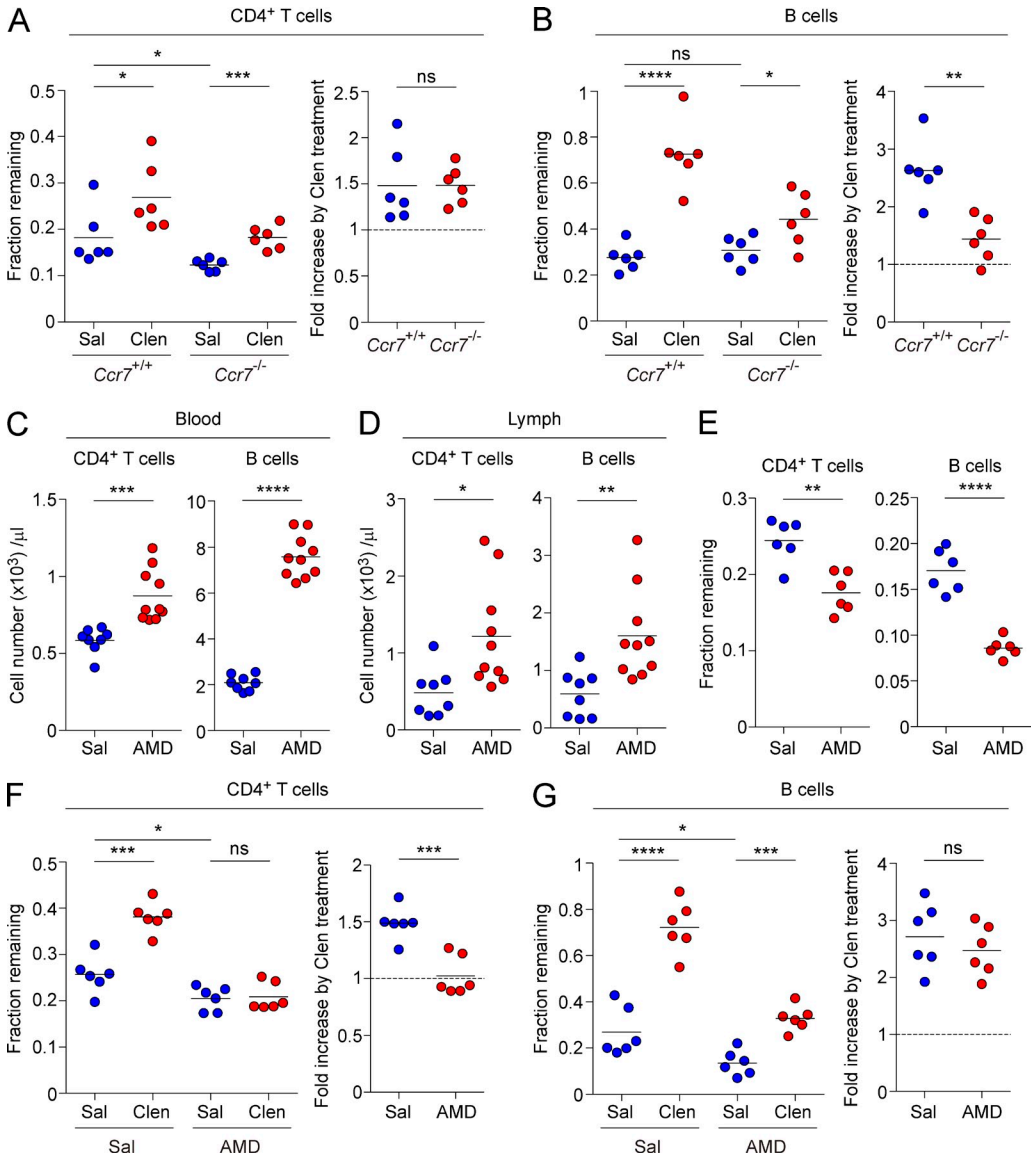

Figure 5. β_2AR-mediated lymphocyte retention depends on coupled chemokine receptors. (A and B) CFSE-labeled *Ccr7*$^{+/+}$ or *Ccr7*$^{-/-}$ spleen cells were transferred to WT recipients, and 2 d later, the mice were treated with integrin-neutralizing antibodies for 18 h during continuous administration 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^{hi}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 β_2ARs suppress T cell–mediated inflammation

Analogous to the action of FTY720, we hypothesized that β_2AR-mediated control of lymphocyte egress might affect the pathogenesis of inflammatory diseases. Indeed, previous studies showed that treatment with β_2AR 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 β_2ARs in T cell–mediated inflammation, we used mouse EAE and delayed-type

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hypersensitivity (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 β_2AR 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, β_2AR-deficient mice developed more severe symptoms compared with β_2AR-sufficient mice (Fig. 6 B), suggesting that physiological levels of inputs through β_2ARs are involved in EAE pathology. The disease induced in β_2AR-deficient mice was not affected by clenbuterol treatment (Fig. 6 C), indicating β_2AR 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 β_2AR agonist started before OVA challenge in the ear attenuated the DTH responses (Fig. 6 D). Additionally, β_2AR-deficient mice exhibited an enhanced OVA-induced DTH response (Fig. 6 E) and unresponsiveness to clenbuterol treatment (Fig. 6 F). Despite increased inflammatory responses, β_2AR 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 β_2ARs act to attenuate T cell–mediated inflammation. Although stimulation of β_2ARs had some impacts on the differentiation of T helper cells in cultures (Sanders, 2012), our results obtained from β_2AR-deficient mice suggest that physiological inputs through β_2ARs might play a minimal role in antigen-induced T cell differentiation in vivo.

Signals through β_2ARs inhibit LN egress of antigen-primed T cells

As an approach to reveal the role of β_2AR-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 β_2AR 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 β_2AR 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 β_2AR-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 β_2AR 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 $CD44^{hi}CD62L^{hi}$ central memory OT-II T cells, but not $CD44^{hi}CD62L^{lo}$ 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), $CD44^{hi}CD62L^{hi}$ OT-II T cells had higher expression of CCR7, whereas CXCR4 was more abundantly expressed in $CD44^{hi}CD62L^{lo}$ OT-II T cells (Fig. 7 D). The expression of β_2AR 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 β_2ARs might contribute to the preferential retention of central memory T cells. These findings suggest that stimulation of β_2ARs in the course of T cell–mediated inflammation selectively inhibits egress of central memory T cells from LNs.

To examine the effect of β_2AR deficiency on the dynamics of antigen-primed T cells, we transferred β_2AR-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 β_2AR-deficient mice, we found that generation and differentiation of antigen-primed OT-II T cells were not affected by β_2AR deficiency (Fig. 7, F and G; and Fig. S7, D and E). Nevertheless, recruitment of β_2AR-deficient OT-II T cells to the inflamed ear was enhanced compared with β_2AR-sufficient cells (Fig. 7 H and Fig. S7 F). Additionally, β_2AR-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 β_2AR deficiency. Indeed, β_2AR deficiency resulted in more rapid egress of $CD44^{hi}CD62L^{hi}$ 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 β_2ARs in $CD44^{hi}CD62L^{lo}$ effector memory OT-II T cells (Fig. 7 E), their LN egress was not affected by β_2AR deficiency (Fig. 7 J). Because the expression of CCR7 and CXCR4 was comparable between β_2AR-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 β_2ARs expressed on antigen-primed T cells control their dynamics in an inflammatory condition. However, we cannot exclude the contribution of β_2ARs expressed on other cell types, including endothelial cells, in which β_2AR-mediated signals are involved in the expression of adhesion molecules (Scheiermann et al., 2012).

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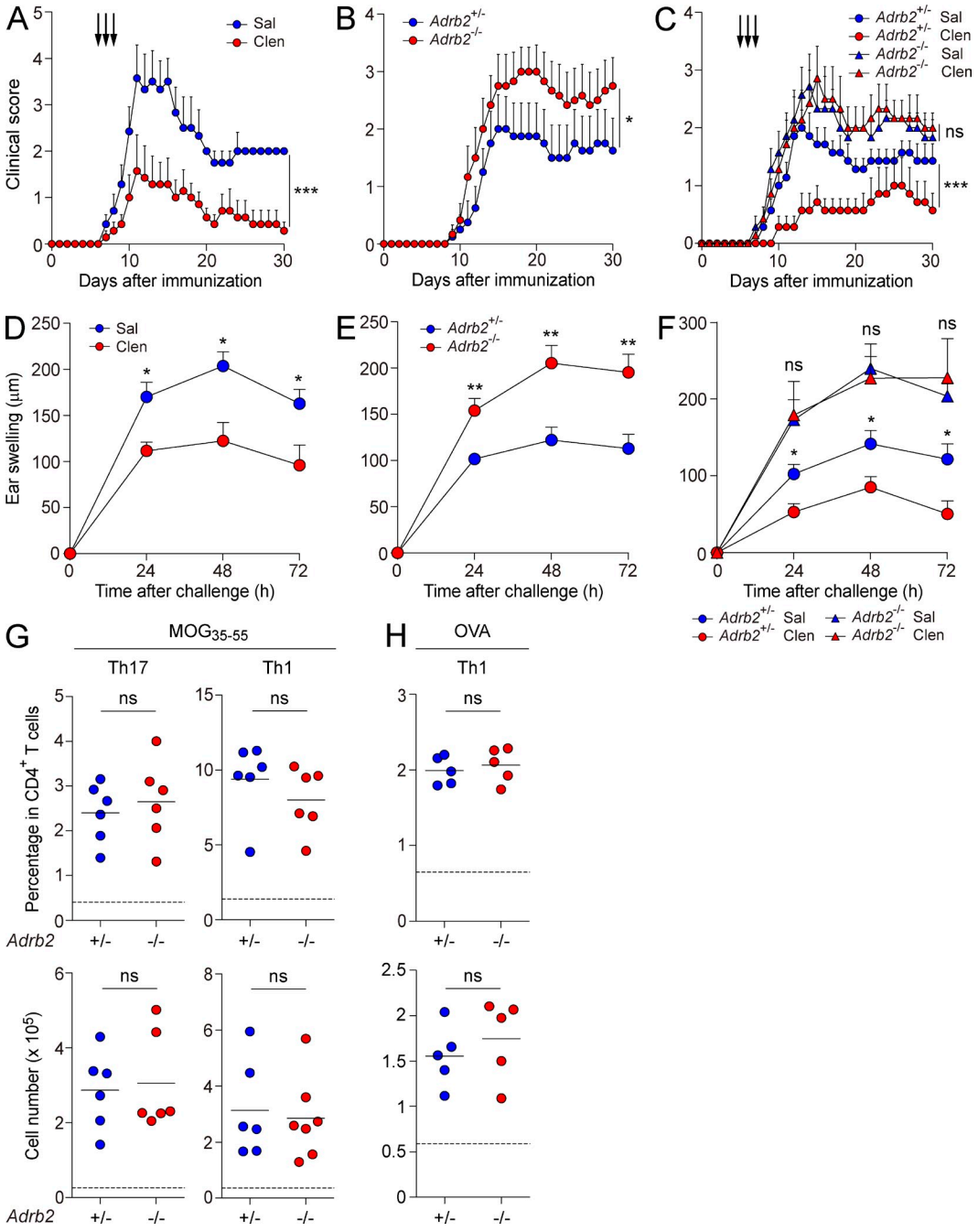

Figure 6. β_2AR-mediated signals attenuate T cell–mediated inflammation. (A) WT mice were immunized with MOG_{35-55} peptide and scored for clinical signs of EAE. Saline ($n = 7$) or clenbuterol (0.4 mg/kg/day, $n = 7$) was injected i.v. for 3 consecutive days at the disease onset. (B) EAE was induced 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/d, 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 ($CD3^+CD4^+IL\text{-}17^+IFN\text{-}\gamma^-$) and/or Th1 ($CD3^+CD4^+IFN\text{-}\gamma^+IL\text{-}17^-$) cells were analyzed in the draining inguinal LNs of *Adrb2*$^{+/-}$ (+/−) and *Adrb2*$^{-/-}$ (−/−) mice 14 d after immunization with MOG_{35-55} peptide (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–C) or unpaired Student's *t* test (D–H).

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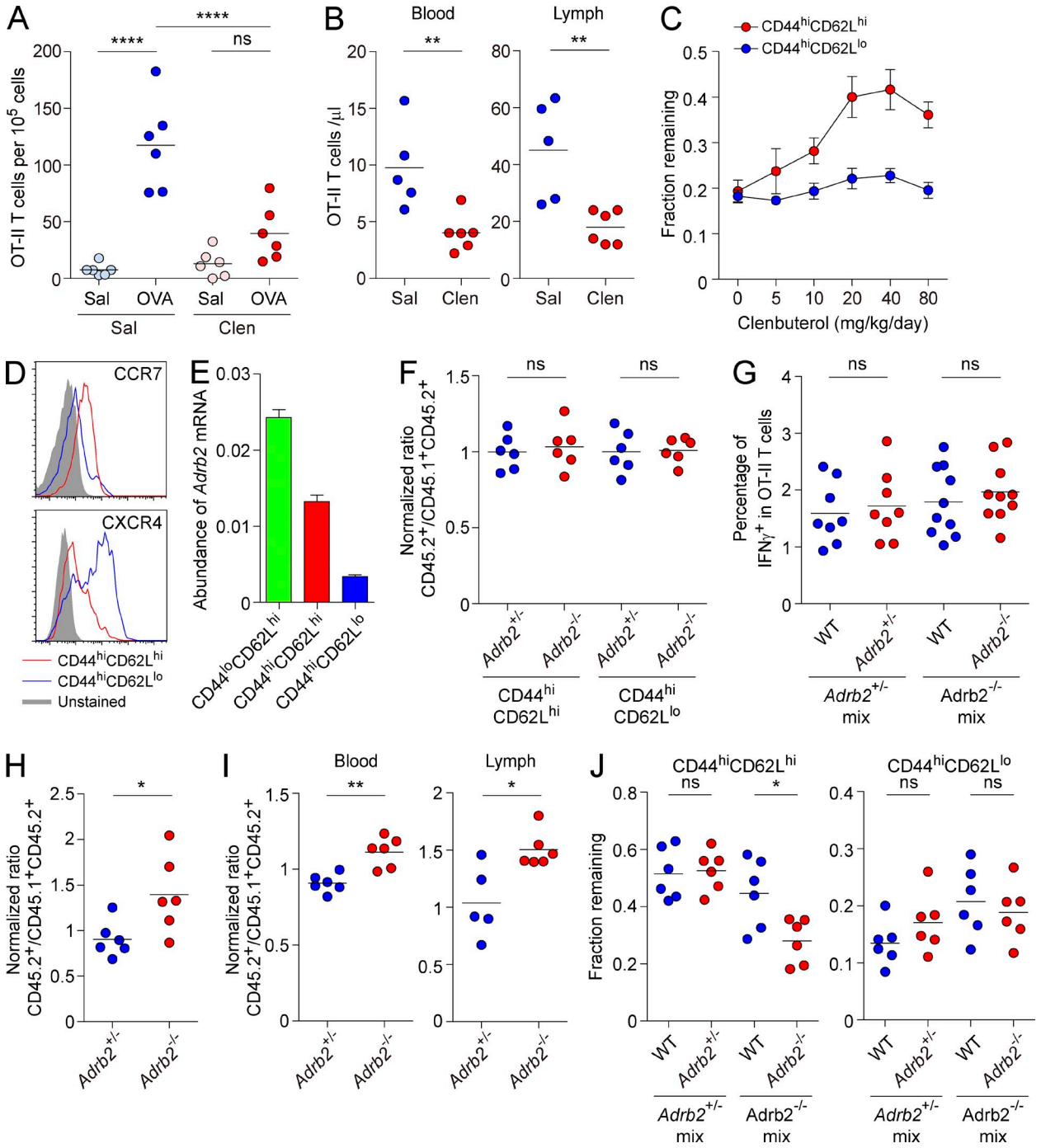

Figure 7. β_2AR-mediated signals inhibit LN egress of antigen-primed T cells. (A–C) WT OT-II T cells (CD45.2$^+$) were transferred into WT mice (CD45.1$^+$), and the recipients were immunized with OVA. 7 d later, the mice were subjected to continuous administration of saline or clenbuterol (40 mg/kg/day). (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 10^5 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 (CD44^{hi}CD62L^{hi}) and effector memory (CD44^{hi}CD62L^{lo}) 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 CD44^{hi}CD62L^{hi} and CD44^{hi}CD62L^{lo} OT-II T cells in the inguinal LNs. (E) *Adrb2* mRNA transcript abundance was measured by quantitative RT-PCR in naive (CD44^{lo}CD62L^{hi}), CD44^{hi}CD62L^{hi}, and CD44^{hi}CD62L^{lo} 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$^+$ WT and CD45.2$^+$ *Adrb2*$^{+/-}$ 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

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DISCUSSION

This study demonstrates that activation of β_2ARs in lymphocytes enhances the responsiveness of retention-promoting chemokine receptors to inhibit lymphocyte egress from LNs. Under physiological conditions, the inputs to β_2ARs 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 β_2ARs in T cells, which might be an additional cause for the lower responsiveness of T cells to augmentation or depletion of β_2AR-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 β_2AR-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 β_2AR-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 β_2ARs 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 β_2AR stimulation on LN retention may be masked by the strong signals evoked by CCR7 in T cells or CXCR4 in B cells, and β_2AR-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 β_2AR 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 β_2AR-mediated signals. During lymphocyte entry from blood to LNs, signals through chemokine receptors induce activation of α4 and αL 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 β_2ARs. This might explain the fact that signal enhancement of CCR7 and CXCR4 by β_2AR 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 β_2ARs might selectively form heteromeric complexes with CCR7 or CXCR4, although a definitive demonstration of their interactions in primary lymphocytes has not been achieved. Because β_2AR stimulation preferentially enhances signals through CCR7 and CXCR4 (Fig. 4, A, D and E), the physical association could be key to functional crosstalk between β_2ARs and chemokine receptors. Heteromerization

dividing observed ratios by the ratio of input populations (F, H, and I). (F) $CD44^{hi}CD62L^{hi}$ and $CD44^{hi}CD62L^{lo}$ OT-II T cells were detected in the draining inguinal LNs. (G) Frequencies of IFN-γ–producing OT-II T cells were measured in the inguinal LNs. (H) Recruitment of OT-II T cells to the ear 12 h after OVA challenge was measured and shown. (I) OT-II T cells in blood and lymph of immunized mice were measured and shown. (J) The immunized mice were treated with integrin-neutralizing antibodies for 14 h, and fractions of $CD44^{hi}CD62L^{hi}$ and $CD44^{hi}CD62L^{lo}$ OT-II T cells remaining in the inguinal LNs were determined as in C. Each symbol represents an individual mouse and bars indicate means (A, B, and F–J). Data are representative of two (D and E) experiments, or pooled from two (A–C, F, and H–J) or three (G) experiments. Sal, saline; Clen, clenbuterol. *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$; ns, not significant. P-values were obtained by one-way ANOVA with Bonferroni's post-test (A) or unpaired Student's *t* test (B and F–J).

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might have allosteric effects on the conformation of chemokine receptors that favor ligand binding or signal transduction, and help converge and synergize their signaling pathways with those of β_2ARs. These possibilities will be tested in future studies to reveal the molecular mechanism of the cross talk between the two classes of GPCRs.

We demonstrated that signals through β_2ARs inhibited LN egress of antigen-primed T cells, reduced their numbers in circulation, and impaired their recruitment to inflamed peripheral tissues (Fig. 7). Although it is possible that these β_2AR-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 studies showed that stimulation of β_2ARs in dendritic cells reduced 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 functions might impair reactivation of central memory T cells at sites of inflammation. We speculate that combination of these β_2AR-mediated effects on immune functions might contribute to the suppression of inflammatory responses.

A recent study showed that inputs from adrenergic nerves induced production of a chemokine CCL20 from blood vessels 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 β_1ARs because treatment with a selective β_1AR antagonist diminished chemokine production by blood vessels and ameliorated disease. Combined with our results, these findings indicate that stimulation of distinct adrenergic receptors β_1 and β_2 might produce opposite outcomes in EAE. Thus, it would be worth exploring the therapeutic potential of β_2AR stimulation, possibly in combination with β_1AR blockade, in T cell–mediated inflammatory diseases.

MATERIALS AND METHODS

Mice. C57BL/6 (B6) mice were obtained from Clea. CD45.1$^+$ and *Ccr7*$^{-/-}$ (*Ccr7*^{tm1Rfor}, MGI: 2180679) mice on a B6 background were purchased from The Jackson Laboratory. *Adrb2*$^{-/-}$ mice on a B6 background were generated as described previously (Hanyu et al., 2012). OT-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 *Adrb2*$^{-/-}$ or CD45.1$^+$ WT mice to generate *Adrb2*$^{-/-}$ 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 fluorophore-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 BD. For analysis of intracellular cytokines, cells were fixed and permeabilized 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 FACSVerse 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$^-$CD23^{hi}CD21^{int}), CD4$^+$ T cells (CD4$^+$CD44^{lo}CD62L^{hi}), and CD8$^+$ T cells (CD8$^+$CD44^{lo}CD62L^{hi}) from the spleen; naive OT-II T cells (CD4$^+$Vα2$^+$ CD44^{lo}CD62L^{hi}) from the spleen of OT-II mice; and OT-II T cells (CD4$^+$Vα2$^+$CD45.2$^+$) with a central memory (CD44^{hi}CD62L^{hi}) or effector memory (CD44^{hi}CD62L^{lo}) phenotype from the draining LNs of OVA-immunized mice that had received OT-II T cells.

Constructs and retroviral transduction. The MSCV2.2 retroviral vectors 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 CXCR5 was also cloned into this vector. Mouse β_2AR was cloned from splenic cDNA and inserted together with a Myc tag at the amino terminus into the MSCV2.2 vector containing Thy-1.1 reporter 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 β_2AR and Flag-tagged CCR7, CXCR4, CXCR5, or S1PR1. The transduced 2PK-3 cells (8×10^6 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 precleared 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-P^{SQ} 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, 10^7 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 PAK1 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 β_2ARs 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 Fluo-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

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objectives (100× oil immersion with 1.40 NA). Co-localization of fluorescent signals was analyzed with a plug-in 'RG2B Colocalization' for ImageJ software.

Quantitative RT-PCR. Total RNA was extracted with a Nucleospin RNA kit (Macherey-Nagel) from FACS-sorted 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 *Adrb2* transcripts: 5′-GTACCGTGCCACCCACAAGA-3′ (forward) and 5′-CCC-GGGAATAGACAAAGACCATC-3′ (reverse).

Transwell migration assay. Spleen cells from WT and *Adrb2*$^{-/-}$ 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 CO_2, lymph was drawn from the cysterna chyli using a fine microcapillary pipette (Matloubian 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 β_2AR 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 surgically implanted s.c. in the back of mice to continuously administer the drugs up to 24 h. Entry blockade was performed 1 h after implantation of the pumps. For depletion of adrenergic nerves, mice were injected i.p. with 100 mg/kg 6-OHDA (Sigma-Aldrich) dissolved in saline containing 0.01% antioxidant ascorbate on days −7 and −5 and 200 mg/kg on day −3 (Grebe et al., 2010). For short-term transfers, mutant or matched control spleen cells were labeled with 0.5 µM CFSE in DMEM containing 1% FBS for 10 min at 37°C. Each recipient mouse received 2×10^7 WT or *Adrb2*$^{-/-}$ cells, or 4×10^7 *Ccr7*$^{-/-}$ cells to overcome a defect in LN entry caused by CCR7 deficiency (Förster et al., 1999). After 2 d of equilibration, entry blockade was performed.

Lymphocyte entry to LN. To test the effect of β_2AR stimulation, 3×10^7 CD45.1$^+$ WT spleen cells were transferred to CD45.2$^+$ mice that had received i.v. injection of saline, clenbuterol (0.4 mg/kg), or salbutamol (12 mg/kg) immediately before cell transfer. To assess the effect of β_2AR deficiency, CD45.2$^+$ *Adrb2*$^{+/-}$ or *Adrb2*$^{-/-}$ and CD45.1$^+$ WT spleen cells were mixed at a ratio of 50:50, labeled with 0.1 µM CFSE in DMEM containing 1% FBS for 15 min at 37°C and transferred to CD45.2$^+$ mice (4×10^7 cells per mouse). 90 min after cell transfer, LNs were collected and subjected to flow cytometry.

EAE. On day 0, mice were immunized s.c. with 100 µg MOG_{35-55} peptide (BEX) emulsified in CFA (Sigma-Aldrich) at the base of the tail. The mice received i.v. 100 ng pertussis toxin (List Laboratories) on days 0 and 2. The clinical symptoms were scored daily as follows: 1, limp tail or abnormal gait; 2, partial hind limb paralysis; 3, complete hind limb paralysis; 4, forelimb weakness; 5, death. For analysis of effector T cell generation, the inguinal LNs were collected on day 14 and disrupted by passage through a 40-µm cell strainer (BD). Cells were stimulated with 50 ng/ml phorbol 12-myristate 13-acetate (Sigma-Aldrich) plus 1 µM ionomycin (Sigma-Aldrich) in the presence of brefeldin A (Sigma-Aldrich) for 6 h and stained for intracellular cytokines as described above. Because initial signs of EAE were reproducibly observed on day 7 or 8 in our particular set of experiments, treatment with clenbuterol (0.4 mg/kg/d, i.v.) was started on day 6, aiming at inhibiting entry of pathogenic T cells into the central nervous system in the early phase of disease.

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 OVA challenge into the ear and left for 3 d. Thickness of 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, 2×10^5 CD45.2$^+$ WT OT-II T cells or mixtures of CD45.2$^+$ *Adrb2*$^{+/-}$ or *Adrb2*$^{-/-}$ plus CD45.1$^+$CD45.2$^+$ WT OT-II cells (2×10^5 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 Dispase I (Roche) in RPMI containing 1% FBS for 1 h at 37°C to remove the epidermis. The dermis was cut into pieces and digested with 0.3 mg/ml Liberase TL (Roche) and 0.05% 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 ED_{50} 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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