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EphB2 and EphB3 play an important role in the lymphoid seeding of murine adult thymus

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ABSTRACT

Adult thymuses lacking either ephrin type B receptor 2 (EphB2) or EphB3, or expressing a truncated form of EphB2, the forward signal-deficient EphB2LacZ, have low numbers of early thymic progenitors (ETPs) and are colonized in vivo by reduced numbers of injected bone marrow (BM) lineage-negative (Lin^-) cells. Hematopoietic progenitors from these EphB mutants showed decreased capacities to colonize wild type (WT) thymuses compared with WT precursors, with $EphB2^{-/-}$ cells exhibiting the greatest reduction. WT BM Lin^- cells also showed decreased colonizing capacity into mutant thymuses. The reduction was also more severe in $EphB2^{-/-}$ host thymuses, with a less severe phenotype in the EphB2LacZ thymus. These results suggest a major function for forward signaling through EphB2 and, to a lesser extent, EphB3, in either colonizing progenitor cells or thymic stromal cells, for in vivo adult thymus recruitment. Furthermore, the altered expression of the molecules involved in thymic colonization that occurs in the mutant thymus correlates with the observed colonizing capacities of different mutant mice. Reduced production of CCL21 and CCL25 occurred in the thymus of the 3 EphB-deficient mice, but their expression, similar to that of P-selectin, on blood vessels, the method of entry of progenitor cells into the vascular thymus, only showed a significant reduction in $EphB2^{-/-}$ and $EphB3^{-/-}$ thymuses. Decreased migration into the $EphB2^{-/-}$ thymuses correlated also with reduced expression of both ephrinB1 and ephrinB2, without changes in the EphB2LacZ thymuses. In the $EphB3^{-/-}$ thymuses, only ephrinB1 expression appeared significantly diminished, confirming the relevance of forward signals mediated by the EphB2-ephrinB1 pair in cell recruitment into the adult thymus.
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Introduction

The thymus, a lymphoid organ responsible for the functional maturation of T lymphocytes, does not contain self-renewing lymphoid progenitors [1], and neither the nature of the progenitors colonizing the thymus nor the underlying molecular mechanisms that govern thymic cell seeding are conclusively known. These mechanisms seem to be distinct during fetal life, when lymphoid progenitors migrate from the fetal liver to an avascular thymic primordium, and in the adult thymus, whose progenitor cells are recruited from the bone marrow (BM) and reach the organ through the blood vessels of the cortico-medullary border [1]. Thus, it has been suggested that certain chemokines (CCL19, CCL21, CCL25, and CXCL12) and adhesion molecules (α4, α5, and α6 integrins, CD44, ICAM-1, VCAM-1) play important roles in the migration to the fetal thymus [2–5]. In contrast, largely P-selectin, CLL25, CCR9, and CCR7 seem to be involved in the colonization of the adult thymus [6–8]. More recently, other molecules have been reported to be implicated in thymic cell recruitment, including the *Caenorhabditis elegans* Ced-5, mammalian DOCK180 and *Drosophila melanogaster* myoblast city (CDM) family of scaffolding members, dedicator of cytokinesis 2 (DOCK2) and DOCK180 [9], the polysialic acid [10] sphingosine-1 phosphate, which mediates progenitor cell egress from the BM [11], semaphorins, and ephrin receptors (Ephs) [12, 13].

Ephs and their ligands, ephrins, are the largest family of tyrosine kinase receptors present in animal cells. Both Ephs and ephrins are subdivided into 2 families, A and B, on the basis of their gene sequence similarities and ligand binding [14]. EphA, which includes 10 members, binds GPI-anchored ephrinA ligands (6 members), and EphB (6 members) interacts with ephrinB transmembrane proteins (3 members). Each Eph can bind several ephrins, and vice versa, and both receptors and ligands transmit intracellular signals, termed forward and reverse signals, respectively [14]. The system is, therefore, very plastic, exhibiting different affinities and expression patterns and determining numerous specific cell-to-cell interactions, which allow these molecules to play a role in a large number of cell functions in different biologic models. We, and others, have demonstrated the relevance of Eph/ephrins in thymus biology [15]. EphB2 and EphB3, the Eph kinases analyzed in the present

Abbreviations: BM = bone marrow, CLP = common lymphoid progenitor, ELP = early lymphoid progenitor, ETP = early thymic progenitor, HSC = hematopoietic stem cell, KO = knockout, Lin = lineage, MPP = multipotent progenitor, TEC = thymic epithelial cell, WT = wild type

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study, and their main ligands, ephrinB1 and ephrinB2, are expressed in both developing thymocytes and thymic stromal cells [16]. EphB2- and EphB3-deficient thymuses show important alterations in the thymic epithelial cell (TEC) network, with modified distribution of the epithelial cell subsets, appearance of cytokeratin-free areas, and delayed maturation of TEC progenitors [17]. In contrast, only slight changes occur in thymocyte differentiation, which largely affects the double negative cell compartment [16]. However, when an imbalance of Eph/ephrin-mediated signals was "forced" in chimeric SCID mice, profound changes occurred in the proportions of distinct thymocyte subsets [18]. Likewise, the deletion of ephrinB1 and/or ephrinB2 in either thymocytes or TECs profoundly affects both TEC populations and thymic histologic features but has little effect on T-cell development [19, 20].

All deficient thymuses exhibited marked hypocellularity that appeared to be associated with increased apoptosis of both thymocytes [16] and TECs [21]. However, defects in the entry of lymphoid progenitors into the thymus could also be implicated, because these molecules have been reported to be involved in the cell migration of distinct immune cells [22–29]. Moreover, we demonstrated the participation of EphB2 in both thymic colonization by BM progenitor cells and in the intrathymic migration of developing T cells [13]. These studies were performed in in vitro reconstitution assays using organ cultures of fetal thymic lobes, a model that, as mentioned, presents some important differences to the in vivo colonization of the adult thymus. Accordingly, we have extended these results in the present study, demonstrating a role for EphB2 and EphB3 in the in vivo colonization of the adult thymus by BM lineage-negative (Lin^-) progenitor cells and the relevance of the thymic microenvironment in the process.

MATERIALS AND METHODS

Mice

EphB-deficient mice, including EphB2 and EphB3 single mutants and EphB2LacZ mice generated in a CD1 background, were kindly provided by Dr. Mark Henkemeyer (University of Texas Southwestern Medical Center, Dallas, TX, USA) to establish our own colony at the Complutense University Animal Care Facilities (Madrid, Spain). The EphB2LacZ mice express a truncated EphB2 molecule fused to β-galactosidase capable of stimulating ephrinB-expressing cells but unable to transmit EphB2 forward signals. EphB-mutant descendants from heterozygous parents were genotyped using PCR before being used in all the experiments. Wild type (WT) mice were EphB knockout (KO) littermates. After backcrossing for several generations, we established 3 subcolonies according to their CD45 haplotype expression (CD45.1, CD45.2, or CD45.1+2). EphB-deficient mice are viable and fertile and show no apparent phenotypic abnormalities. The litter sizes from the WT ($EphB^{+/+}$) pair matings were not different from those of the $EphB^{-/-}$ pair litters. All mice were bred and maintained under specific pathogen-free conditions at the animal housing facilities of the Complutense University of Madrid.

Cell suspensions, antibodies, and flow cytometry

BM cells and thymocytes were collected, as previously described [13]. In brief, the BM cells were obtained by flushing femur, tibia, and humerus with RPMI/10% FBS/5 mM EDTA and a subsequent Histopaque-1083 (Sigma-Aldrich, St. Louis, MO, USA) density gradient centrifugation. The cells were then incubated with the biotinylated Lin antibody cocktail (BD Pharmingen, San Jose, CA, USA), followed by a bead-conjugated anti-biotin antibody (Miltenyi Biotec, Bergisch Gladbach, Germany). The BM cell suspensions were highly enriched in Lin^- cells by magnetic negative selection in an AutoMACS (Miltenyi Biotec), reaching a minimum purity of 95%. Thymocyte cell suspensions were prepared by gently pressing isolated thymuses. The cells were washed in cold medium (RPMI/5% FBS) and subsequently stained with specific antibodies.

The antibodies in the Lin cocktail (BD Pharmingen) included anti-B220 (clone RA3–6B2), anti-CD11b (M1/70), anti–Gr-1 (RB6-8C5), anti–Ter-119 (Ter-119), and anti-CD3 (145-2C11), either biotinylated or allophycocyanin (APC) conjugated. Additional antibodies used in the procedures were anti-CD4 purified (GK1.5), anti-CD25 (PC61), anti-CD44 (IM7), anti-CD45.1 (A20), anti-CD45.2 (104), anti-CCR7 (4B12), anti-CCR9 (242503), anti–P-selectin glycoprotein ligand 1 (PSGL1) (2PH1), anti–c-Kit (2B8), anti-Flt3 (A2F10), anti–IL-7 receptor subunit α (IL-7Rα) (A7R34), and anti-Sca1 (D7). Antibodies were purchased from BD Pharmingen, BioLegend (San Diego, CA, USA), eBioscience (San Diego, CA, USA), or R&D Systems (Abingdon, UK) and conjugated with fluorescein isothiocyanate, phycoerythrin, peridinin chlorophyll protein-Cy5.5, or APC. Cell staining was performed with specific antibodies in PBS plus 1% FBS for 20 min, according to routine procedures. Flow cytometric analyses were performed using a FACSCalibur device (BD Biosciences, San Jose, CA, USA) equipped with CellQuest software at the Cytometry and Fluorescence Microscopy Centre (Complutense University). The analyses were performed using FCS Express software (De Novo Software, Los Angeles, CA, USA).

The early thymic progenitor (ETP) subpopulation was obtained by analyzing in the Lin APC-negative cell population, the percentage of $c\text{-}Kit^+$ cells within the $CD44^+CD25^-$ subset. The hematopoietic stem cell (HSC), multipotent progenitor (MPP), early lymphoid progenitor (ELP), and common lymphoid progenitor (CLP) proportions were determined in BM cell suspensions to analyze the expression of Sca1 and c-Kit in different regions, defined according to their differential Flt3 and IL-7Rα expression. For the analysis of the BM subsets expressing receptors for either chemokines or P-selectin, we used a similar procedure, in which anti-IL-7Rα antibody was substituted with anti-CCR7, anti-CCR9, or anti-PSGL1 antibodies.

Intravenous transfers

Lin^- BM cells were prepared as described. For single i.v. transfers in all the assayed combinations, 10^6 Lin^- BM donor cells were injected i.v. in the tail of anesthetized 7–9-wk-old host mice, both with different CD45 haplotypes (CD45.1 or CD45.2). The mice were anesthetized with a mix of ketamine and xylazine by i.p. injection before the cell transfers were made, to avoid the mice experiencing any pain during the tail injection, according to institutional guidelines and approval by the Complutense University Ethical Committee for Animal Experimentation. For competitive colonization assays, a similar protocol was used: 10^6 Lin^- BM cells of each donor (WT mice [CD45.2] and EphB-deficient mice [CD45.1]) were mixed and injected in 7–9-wk-old heterozygous host mice (CD45.1+2). In addition, we tested, as previously reported [30, 31], the presence of donor cells in the BM of host mice, confirming that, apart from the thymus, they had also seeded in this organ. As previously described [32], to prevent rejection, the host mice were given 0.1 mg of anti-CD4 (GK1.5) antibody the day before BM cell transfer and every 4 d thereafter. Equivalent chimerism was observed between CD45.1 and CD45.2 donor cells in the BM (data not shown) of the recipient mice in the competition colonization experiments, confirming that rejection had not occurred. The host thymuses were analyzed using flow cytometry to detect CD45 chimerism, 3 wk after cell injection.

Immunofluorescence microscopy and tissue quantification

For immunofluorescence microscopy and tissue quantification, 6 μm-thick cryosections from either WT or EphB-deficient mice (aged 7–9 wk) were fixed in acetone for 10 min and air-dried. The slides were then incubated for 1 h at room temperature with primary antibodies specific for either mouse P-selectin (dilution 1/50), CCL21 (1/100), CCL25 (1/25), ephrinB1 (1/20), ephrinB2 (1/20) (R&D Systems), panendothelial cell antigen (1/100) (MECA32,

BioLegend), and laminin (1/100) (Sigma-Aldrich). Alexa 488 anti-goat IgG, Alexa 488 anti-rabbit, and Alexa 594 anti-rat IgG (Molecular Probes, Eugene, OR, USA) were used as secondary antibodies and incubated for 45 min. The sections were mounted in Antifade Prolong Gold (Molecular Probes) and photographed in a Zeiss Axioplan microscope (objectives: 10× ∞/0.30 Plan-Neofluar, 20× ∞/0.50 Plan-Neofluar, 40× ∞/0.17 Plan-Neofluar; Carl Zeiss, Jena, Germany) equipped with a Spot2 digital camera. The expressed results are the mean values of a minimum of 10 nonoverlapping regions from different serial thymic sections of at least 4 different mice per genotype. The resultant images were quantified using Adobe Photoshop CS5 (Adobe Systems, San Jose, CA, USA). The quantitative fluorescence analyses were performed by calculating the number of specifically stained pixels, divided by the analyzed area in pixels and multiplied by 100 to obtain the percentage of stained pixels in every picture.

Electron microscopy

For electron microscopy, thymuses isolated from adult WT and EphB-deficient mice were fixed in 2.5% glutaraldehyde/0.1 M sodium cacodylate at 4°C for 3–5 h and embedded in Durcopan resine (Fluka AG, St. Gallen, Switzerland), as previously described [17]. Semithin sections were stained with toluidine blue for 5 min, dehydrated in 70–100% ethanol (5 min) (Panreac, Barcelona, Spain) and in xylene baths (5 min), mounted in DPEX (Panreac), and examined by light microscopy to identify the blood vessels in the thymic cortex and corticomedullary border for additional ultrastructural analysis. Ultrathin sections were double stained with uranyl acetate and lead citrate and studied in a JEOL 10.10 electron microscope (JEOL USA, Peabody, MA, USA) at the Cytometry and Fluorescence Microscopy Centre of Complutense University (Madrid, Spain).

Statistical analysis

The data are presented as the mean ± SD. The statistical significance of the data was assessed using Microsoft Excel 2010 (Redmond, WA, USA) using Student's *t* test and represented as NS ($P \geq 0.05$), $P < 0.05$, $P < 0.01$, and $P < 0.005$.

RESULTS

Lack of EphB2 or EphB3 affects the subpopulation of ETPs

To evaluate the possible contribution of colonizing T-cell precursors to the thymic hypocellularity observed in EphB-deficient thymuses [16], we first evaluated the ETP percentages in the thymus of adult EphB2 and EphB3 KO mice. In both EphB2- and EphB3-deficient mice, the percentages of ETP cells, defined as Lin^- $cKit^{hi}$ $CD44^+CD25^-$ cells [6, 33], decreased severely compared with those observed in the WT mice (**Fig. 1**). This reduction was close to 50%, highlighting the importance of EphB receptors for the complex processes that govern the arrival of lymphoid progenitors into the adult thymus. The proportions of ETP cells in EphB2LacZ mice, unable to generate forward signals in the EphB-expressing cells but able to activate the reverse signaling transmitted by the expressed ephrinB ligands, also decreased, compared with the control mice, but less importantly than in both EphB2- and EphB3-deficient thymuses (Fig. 1). These results indicated a role for reverse signaling in control of the ETP cell number but emphasized the relevance of the forward signals transmitted by the EphB receptors.

EphB2 signaling affects the development of early BM hematopoietic progenitor cells

Several events could be contributing to the observed reduction of ETP percentages in EphB KO mice, including the existence of

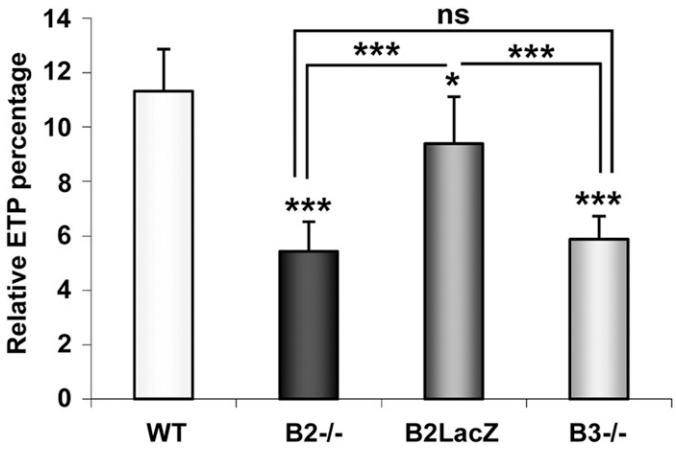

Figure 1. Reduced percentages of ETP cells in EphB-deficient mice. The percentages of ETP cells (Lin^-c-$Kit^{hi}CD44^+CD25^-$ cells) were determined by FACS in adult WT and EphB-deficient thymuses, gating the $CD44^+CD25^-$ cell population within the Lin^- cell subset and showing the values of c-Kit within the subset. The percentage of ETP cells was significantly lower in EphB2- or EphB3-deficient thymuses than in WT thymuses. Mice expressing a truncated form of EphB2, EphB2LacZ, showed increased values compared with those of EphB-deficient mice, but they did not reach the WT values. Data (mean ± SD) are representative of ≥ 5, sex-matched, 7–9-wk-old mice from each group in the indicated mouse strains. *P* values from Student's *t* test statistical analysis are indicated as NS ($P \geq 0.05$), *$P < 0.05$, and ***$P < 0.005$.

a lower number of lymphoid progenitors with the capacity to colonize the EphB-deficient adult thymus, alterations in the BM exit of these progenitor cells, and in their trafficking into the adult thymus. We first studied the possible variations in the 4 main hematopoietic progenitor cell subsets defined by the expression of Sca1, c-Kit, IL-7Rα, and Flt3 cell markers [1, 34], using Lin^- BM cell suspensions from either adult EphB-deficient or WT mice (**Fig. 2**).

We observed a significantly reduced proportion of the 4 assayed subsets in the EphB2 KO mice (Fig. 2). Remarkably, the lack of EphB3 did not affect any of the studied subsets. In the case of EphB2LacZ mice, the most primitive progenitor cell subsets, HSCs (Lin^-Sca1^+c-$Kit^+Flt3^-IL\text{-}7R\alpha^-$ cells) and MPPs (Lin^-Sca1^+c-$Kit^+Flt3^+IL\text{-}7R\alpha^-$ cells) underwent significant reductions, just as found in EphB2 KO mice but not in the ELPs (Lin^-Sca1^+c-$Kit^+Flt3^+IL\text{-}7R\alpha^+$ cells) and CLPs (Lin^-Sca1^{lo} c-$Kit^{lo}Flt3^+IL\text{-}7R\alpha^+$ cells). These results again suggested the relevance of EphB2 in the development of hematopoietic progenitors in the mouse BM.

Thymus colonization capacity is impaired in the EphB-deficient BM precursor cells

Together with changes in the proportions of BM hematopoietic progenitors, the low numbers of ETPs found in the EphB KO thymuses could also have resulted from defects in the lymphoid progenitors able to colonize the thymus: defects in their numbers or in their ability to reach the thymus parenchyma. Because different evidence has suggested that lymphoid progenitors capable of colonizing the thymus express specific receptors that allow their attraction to the thymic parenchyma by chemokines and other molecules [1], we examined the expression of CCR7,

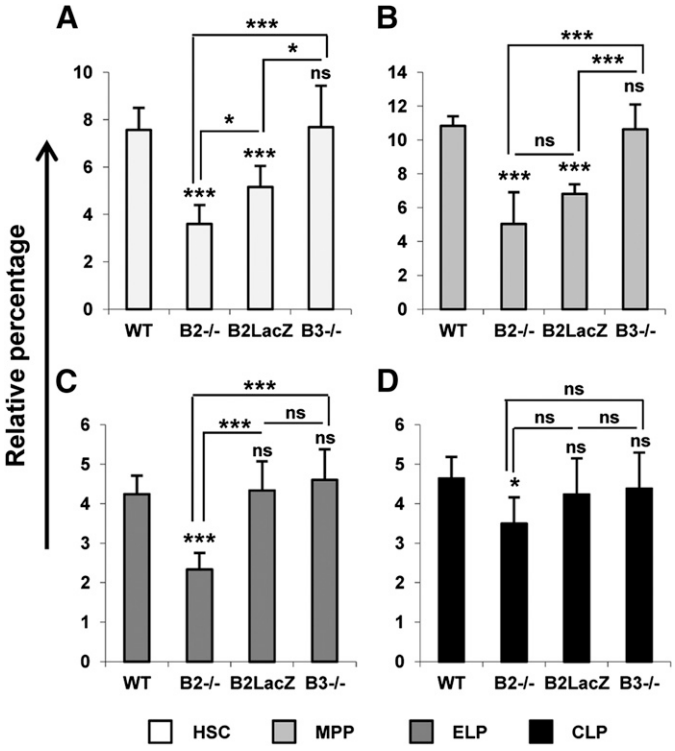

Figure 2. Percentages of BM progenitor cell subpopulations in WT and EphB-deficient mice. Hematopoietic progenitor cell subsets were defined by the expression of Sca1, c-Kit, Flt3, and IL-7Rα cell markers: HSC (A), MPP (B), ELP (C), and CLP (D). The percentages of all tested progenitor cells were significantly lower in the EphB2$^{-/-}$ BM than in the WT BM, and the EphB3$^{-/-}$ mice showed similar values to those from WT BM (A–D). The EphB2LacZ mice presented with a significantly lower percentage of the earliest hematopoietic precursor cells, HSC and MPP (A and B), but not those of ELP and CLP (C and D). Data (mean ± SD) are representative of 5 sex-matched mice from each group in the indicated mouse strains. *P* values from Student's *t* test statistical analysis are indicated as NS ($P \geq 0.05$), *$P < 0.05$, and ***$P < 0.005$.

CCR9, and PSGL1 in the HSC, MPP, ELP, and CLP subsets of adult BM. MPPs and ELPs were studied as a single cell population. We did not find statistically significant variations in the percentages of EphB-deficient progenitor cells expressing CCR7, CCR9, and PSGL1 compared with their WT counterparts (**Fig. 3**).

Next, we performed in vivo colonization assays in which we i.v. injected 10^6 Lin$^-$ BM cells isolated from either EphB-deficient or WT mice to test their capacity to colonize WT host thymuses. Donor (CD45.1) and host (CD45.2) mice expressing different CD45 haplotypes were used to discriminate the exogenous and endogenous thymic cells present in the host thymus 3 wk after the cell injections. As previously described [8, 31, 32], in all colonization experiments, the host mice were treated with anti-CD4 antibody to prevent cell donor rejection. Our results indicated that WT thymuses contained lower proportions of the injected EphB-deficient cells than WT cells (**Fig. 4A**). The proportions were particularly low in the case of mice receiving EphB2 KO Lin$^-$ cells, showing significant differences, not only with respect to the control values, but also compared with WT thymuses colonized by either EphB3 or EphB2LacZ cells, whose proportions were also lower than those of WT Lin$^-$ cells (Fig. 4A). No differences were found between mice receiving EphB2LacZ Lin$^-$ cells and those injected with EphB3-deficient mice (Fig. 4A).

In addition, we tested the ability of these EphB-deficient precursors to reach the WT thymuses in competitive conditions with WT cells injected at the same time (Fig. 4B and C). For this purpose, we injected the same number of Lin$^-$ BM cells isolated from either WT mice (CD45.2) or EphB-deficient mice (CD45.1) into heterozygous WT host mice (CD45.1 plus CD45.2). In these experimental conditions, we could distinguish endogenous WT thymocytes from those derived from either WT or EphB-deficient Lin$^-$ cells. The injected WT cells colonized the WT host thymus normally, but all types of EphB-deficient cells studied appeared in significantly lower proportions than the injected WT Lin$^-$ cells (Fig. 4B and C). Nevertheless, the reduction in migrating mutant cell numbers was more severe, compared with the WT donor cells, in experiments performed in competitive conditions (Fig. 4B and C) than when Lin$^-$ BM cells from either WT or mutant mice were injected alone (Fig. 4A).

Migration of WT BM progenitor cells into EphB-deficient thymuses is profoundly reduced

The relevance of a thymic microenvironment for BM progenitor cell colonization previously claimed [6, 13, 35] was evidenced after i.v. injection of 10^6 WT cell progenitors in EphB-deficient mice. Three weeks later, the number of WT BM Lin$^-$ donor cells was significantly lower in the 3 mutant thymuses than in the WT ones (**Fig. 5**). Although, the 3 EphB-deficient thymuses showed significantly reduced proportions of WT cells, the reduction was particularly severe in the EphB2-deficient thymuses. In contrast, EphB2LacZ mice showed significantly greater proportions of colonizing WT cells than those found in both EphB2- and EphB3-deficient mice, although the values did not reach those observed when WT thymuses were used as hosts in the experiments (Fig. 5). These results suggest the importance of ephrin reverse signaling for thymic colonization and the need for forward signals transmitted through EphB2 and EphB3 receptors.

When we compared both colonization assays, the reduction in thymic immigrant cells was higher in the EphB3$^{-/-}$ thymuses receiving WT donor progenitors (Fig. 5) than in the WT thymuses receiving EphB3$^{-/-}$ progenitor cells (Fig. 4A). In contrast, the proportions of intrathymic donor cells in EphB2LacZ thymuses receiving WT progenitors (Fig. 5) were greater than those observed in WT thymuses colonized by EphB2LacZ precursor cells (Fig. 4A). In the EphB2$^{-/-}$ thymuses that received WT progenitors (Fig. 5), the proportion of intrathymic donor cells was similar to that of WT thymuses colonized by EphB2$^{-/-}$ precursor cells (Fig. 4A), presumably because the donor cell frequency was severely reduced in both cases.

To further evaluate the relative relevance of donor BM progenitors and host stroma, we analyzed thymic colonization, combining differentially EphB-deficient BM progenitor cells and WT and mutant thymic stromas (**Fig. 6**). To achieve this, we selected and crossed for several generations CD45.2 EphB KO mice to generate a new mouse colony that allowed us to

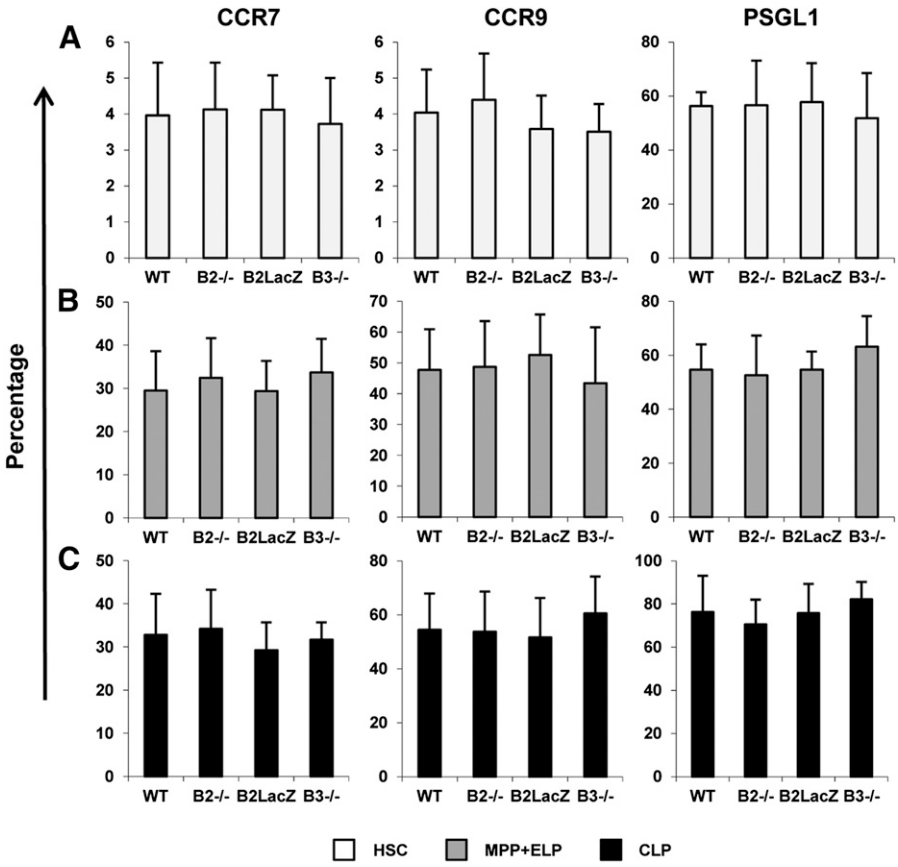

Figure 3. No changes were detected in the proportions of chemokine or P-selectin receptor-expressing progenitor cells of EphB-deficient mice. The expression of CCR7, CCR9, and PSGL1 was analyzed by FACS in 3 groups of BM progenitors defined as (A) HSC ($Lin^-Sca1^+c\text{-}Kit^+Flt3^-$), (B) MPP and ELP ($Lin^-Sca1^+c\text{-}Kit^+Flt3^+$), and (C) CLP ($Lin^-Sca1^{-/low}c\text{-}Kit^{-/low}Flt3^+$). No significant differences were observed ($P \geq 0.05$) between populations isolated from WT and EphB-deficient mice. Data are representative of 4 independent experiments (mean ± SD). The mice were aged 7–9 wk and sex matched. Student's *t* test statistical analysis was performed.

distinguish donor vs. host cells when both lacked EphB receptor expression. We then tested the in vivo capacity of different EphB-deficient precursors to colonize in different altered EphB microenvironments (Fig. 6).

$EphB2^{-/-}$ progenitors showed altered thymic colonization into all the assayed EphB-deficient thymuses compared with their capacity to colonize WT thymuses (Fig. 6A). The lowest levels of EphB2-deficient thymic cells occurred in both EphB2 KO and EphB2LacZ thymuses. In contrast, EphB3-deficient thymuses were colonized slightly better (Fig. 6A). Although in all conditions, the EphB-deficient stromas were more poorly colonized by any mutant BM progenitors than by WT ones, the situation when EphB2LacZ (Fig. 6B) or EphB3 (Fig. 6C) progenitors were injected in EphB2-deficient thymuses was again particularly severe. Accordingly, EphB2-deficient stroma exhibited the lowest capacity to be colonized by any of the 4 types of Lin^- BM precursor cells tested. Both EphB2LacZ and EphB3-deficient thymuses showed a lower capacity to be colonized than did the WT thymuses; however, they contained significantly greater numbers of donor cells than did the EphB2-deficient host thymuses.

EphB-deficient mice present defective expression of P-selectin and chemokines involved in progenitor cell recruitment into the thymus

Because in other experimental models, decreased expression of homing molecules has been accounted for by decreased thymic progenitor receptivity [36], we studied immunohistochemically the expression of P-selectin (**Fig. 7**) and chemokines CCL12 and CCL25 (**Fig. 8**) in the thymus of adult WT and EphB-mutant mice, quantifying their values using software-based analysis. Our results showed a very significant reduction in P-selectin expression compared with the WT thymus in both EphB2- and EphB3-deficient thymuses, but not in the EphB2LacZ mice (Fig. 7A and B). Regarding the chemokines, in both control and mutant thymuses, CCL21 was largely expressed in the thymic medulla, and CCL25 appeared in both the cortex and the medulla (Fig. 8). In addition, all mutant thymuses showed significantly reduced expression of CCL21 and CCL25 compared with the control thymuses, with EphB2-deficient thymuses exhibiting the most severe reduction, particularly of CCL21 expression (Fig. 8A).

We analyzed the structure of the thymic blood vessels (**Fig. 9**), because progenitor cell migration into the adult thymus occurs through them, and it has been reported that mice lacking EphB2 or EphB3 have defective vasculogenesis [37]. Furthermore, we evaluated the expression of CCL21 and CCL25 (Fig. 8), which attract $CCR7^+$ and $CCR9^+$ cells, respectively, into the thymus [1], and of ephrinB1 and ephrinB2, the main EphB2 and EphB3 ligands, which mediate the EphB-mediated lymphoid progenitor transmigration (**Fig. 10**). Using laminin expression as a marker of the thymic vasculature pattern, we observed that the blood vessels of the 3 mutant thymuses studied appeared enlarged and their walls thickened compared with those of the WT thymuses (Fig. 9A–D). By electron microscopy, the blood vessels of the mutant

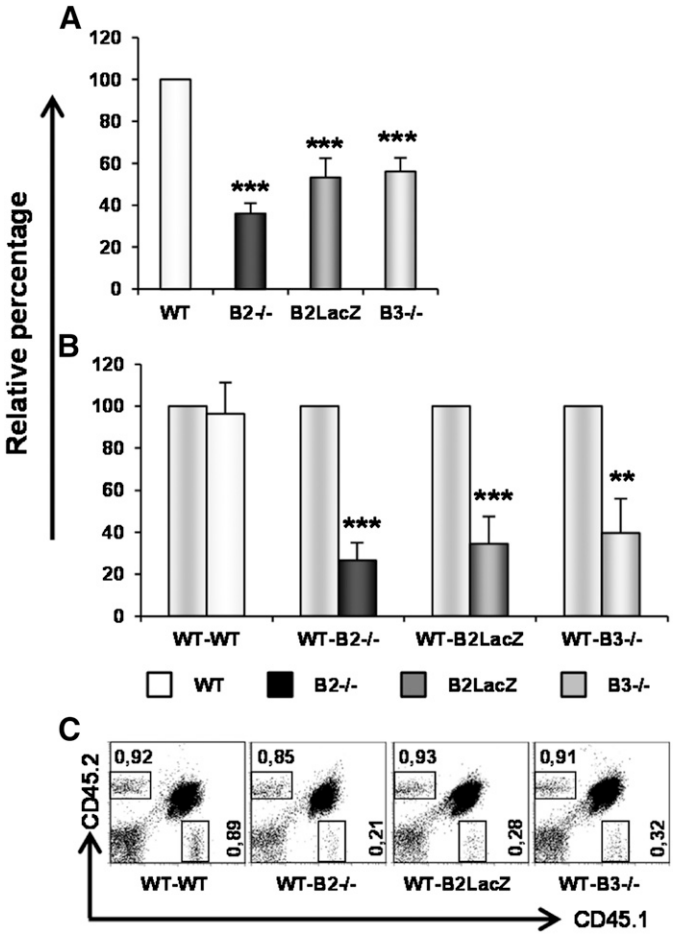

Figure 4. EphB-deficient Lin$^-$ BM cells colonized the WT thymuses poorly. (A) At 3 wk after i.v. injection of BM Lin$^-$ cells into WT host mice, the thymuses were analyzed to determine the relative proportions of donor CD45.1$^+$ cells and endogenous host CD45.2$^+$ thymic cells. Lin$^-$ progenitor cells derived from EphB-deficient BM showed a diminished capacity to enter the thymus. (B) Competitive assays were also performed in which BM Lin$^-$ cells from WT and EphB-deficient mice were injected i.v. in the same WT mouse. Newly EphB-deficient progenitors colonized the host thymus less effectively than did the WT cells. Data are representative of 4 independent experiments (mean ± SD) with 7–9-wk-old, sex-matched mice. In each experiment, the relative percentages with respect to the WT values are shown. *P* values from Student's *t* test statistical analysis are indicated as NS ($P \geq 0.05$), $^{**}P < 0.01$, and $^{***}P < 0.005$. (C) FACS analysis of WT host thymuses (CD45.1 plus CD45.2) after i.v. transfers of WT (CD45.2) and EphB-deficient mice BM Lin$^-$ cells (CD45.1) in competitive adoptive colonization assays. Representative plots are shown.

thymic cortex showed enlarged perivascular spaces that did not appear in the WT thymus (Fig. 9E–H). In addition, high-endothelial vessels of the corticomedullary area of the mutant thymuses showed important reinforcements of their basal membrane that could hinder lymphoid progenitor migration (Fig. 9I–N).

In contrast, in both mutant and WT thymuses, association of CCL21 expression and MECA32$^+$ vascular endothelia was clearly evidenced (Fig. 8C–F). However, CCL25 expression occurred more frequently surrounding the blood vessels (Fig. 8G–J). The quantitative study confirmed a significant decrease in CCL12/MECA32 and CCL25/MECA32 staining in both EphB2- and EphB3-deficient thymuses but not in the EphB2LacZ ones (Fig. 8B). We have also reported that ephrinB expression remained in the thymuses of mice deficient in their EphB2 and EphB3 receptors [16] (Fig. 10), but the quantification of ephrinB staining associated with MECA32$^+$ blood vessels (Fig. 10B–I) demonstrated a significant decrease in ephrinB1 expression in the thymic vascular endothelia of EphB2- and EphB3-deficient mice but not in the EphB2LacZ ones (Fig. 10A). EphrinB2 expression only decreased significantly in the EphB2$^{-/-}$ thymic blood vessels and not in those of EphB3$^{-/-}$ or EphB2LacZ thymuses (Fig. 10A).

DISCUSSION

The trafficking of lymphoid progenitors into the thymus is key to understanding T-cell maturation but has yet to be definitively resolved [1]. Together with the assumed role of chemokines, adhesion molecules, and P-selectin in the process [2, 4, 6–8], our present results have demonstrated the relevance of EphB2 and EphB3 in in vivo progenitor cell recruitment into the adult thymus, confirming our own previous results obtained in an in vitro reconstituted assay using organ cultures of fetal thymic lobes [13].

Ephs and ephrins have been implicated in cell migration in various systems [38], although the in vivo studies are few and have been performed in experimental models very distinct to those we analyzed in the present study. In this context, in vivo infusion of preclustered EphB2-Fc or ephrinB2 proteins in the subventricular zone disturbs the migration of neuronal precursors [39, 40], and disruption of Eph/ephrin signaling reduces

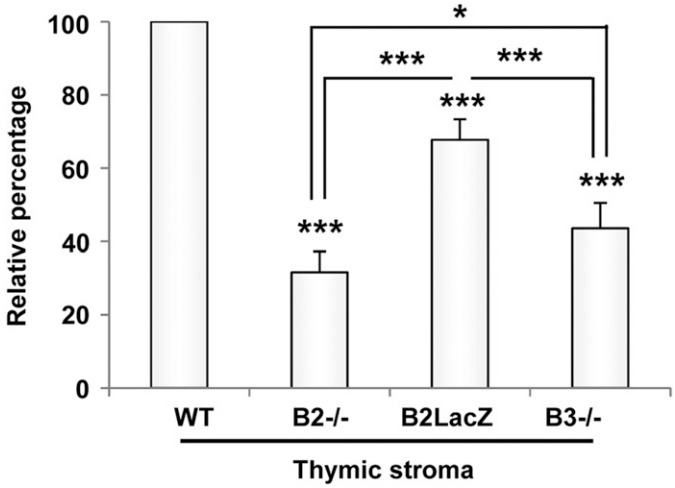

Figure 5. WT BM progenitors show impeded colonization of EphB-deficient thymuses. At 3 weeks after WT BM progenitor cells were injected i.v. in adult EphB KO hosts, a significant reduction was seen in WT cell proportions in the mutant host thymuses; particularly low percentages occurred in EphB2 KO thymuses, with less drastic effects in EphB2LacZ thymuses. Values shown are relative percentages of WT values. Data are representative of 4 independent experiments (mean ± SD). Mice were sex matched and 7–9 wk old. Student's *t* test statistical analysis was done: $^{*}P < 0.05$ and $^{***}P < 0.005$.

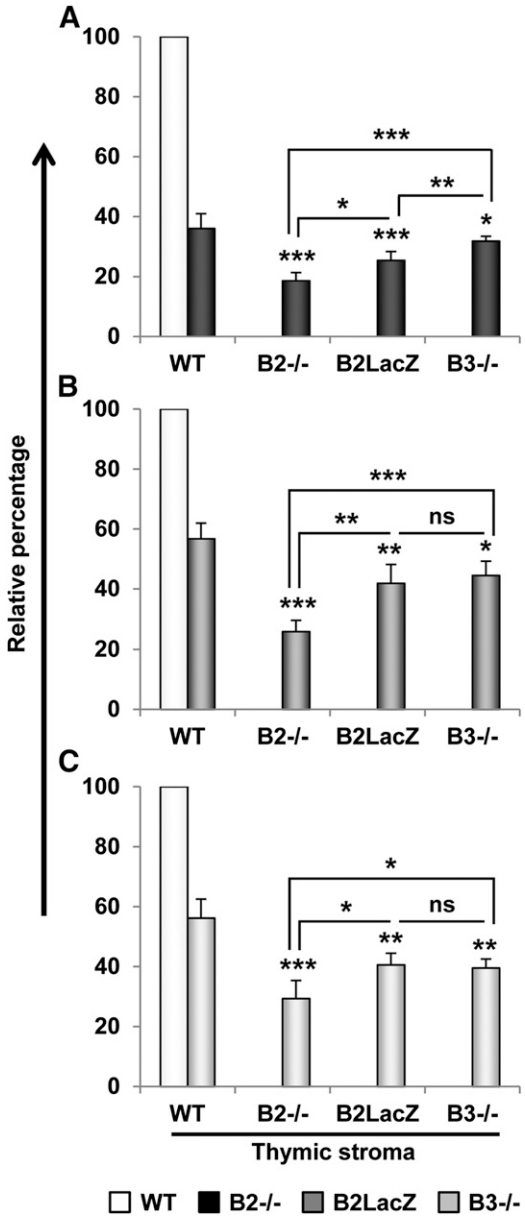

Figure 6. EphB-deficient BM progenitor cells colonize WT and EphB mutant thymuses with different efficiency. EphB2$^{-/-}$ (A), EphB2LacZ (B), and EphB3$^{-/-}$ (C) BM progenitor cells were injected i.v. in either WT or EphB-deficient mice, and their intrathymic presence was evaluated by FACS 3 weeks later. In all cases, the EphB2$^{-/-}$ thymic stroma showed the lowest numbers of WT or EphB-deficient cells. Both EphB2LacZ and EphB3$^{-/-}$ thymuses showed similar homing capability, but always lower than that of WT thymuses, except for EphB2$^{-/-}$ progenitor cells that colonized EphB2LacZ stroma slightly worse than EphB3$^{-/-}$ thymuses. Data shown are related to the percentage of WT colonizing progenitors into WT thymuses (100%) (first column). Data (mean ± SD) are representative of 4 sex-matched mice in each group in the indicated mouse strains. *P* values from Student's *t* test statistical analysis are indicated as NS ($P \geq 0.05$), *$P < 0.05$, **$P < 0.01$, and ***$P < 0.005$.

the efficiency of Cajal-Retzius cell dispersion in the cortex induced by CXCL12 produced by meninges [41]. Furthermore, EphA2 modulates the accumulation of T cells and dendritic cells in the lungs of EphA2$^{-/-}$ mice injected with *Mycobacterium tuberculosis* [42], and ephrinA reverse signaling promotes the migration of cortical interneurons from the medial ganglionic eminence. In EphA4$^{-/-}$ mice and ephrinA2-deficient mice, interneuronal migration is delayed [43].

The reduced numbers of both ETPs and injected migrating hematopoietic cells into the adult thymus can be attributed to several processes. Thus, our study found lower numbers of hematopoietic progenitor cells in the BM of EphB2 KO and EphB2LacZ mice than in the WT BM, defects in the migration into the thymus of both WT and mutant progenitor cells, and altered expression of molecules involved in both thymic recruitment and transmigration processes throughout the blood vessels in the mutant thymuses.

EphB2- but not EphB3-deficient mice exhibited reduced proportions of the 4 BM hematopoietic cell subsets studied

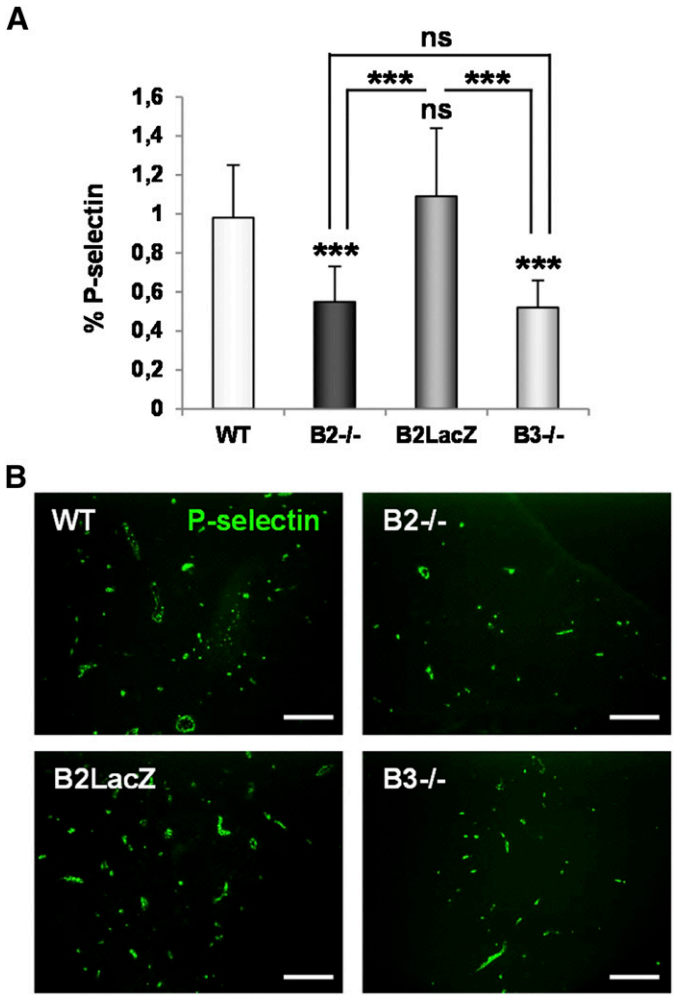

Figure 7. Reduced expression of P-selectin in both EphB2$^{-/-}$ and EphB3$^{-/-}$ thymuses but not in EphB2LacZ ones. (A) The analysis of serial thymic cryosections of the different EphB-deficient mice showed a lower expression of P-selectin in both EphB2- and EphB3-deficient mice. In contrast, the EphB2LacZ thymuses showed P-selectin expression similar to that of WT mice. (B) P-selectin expression in representative thymic sections of WT and EphB-deficient mice are shown. *P* values from Student's *t* test statistical analysis are indicated as NS ($P \geq 0.05$) and ***$P < 0.005$. Scale bars, 200 μm.

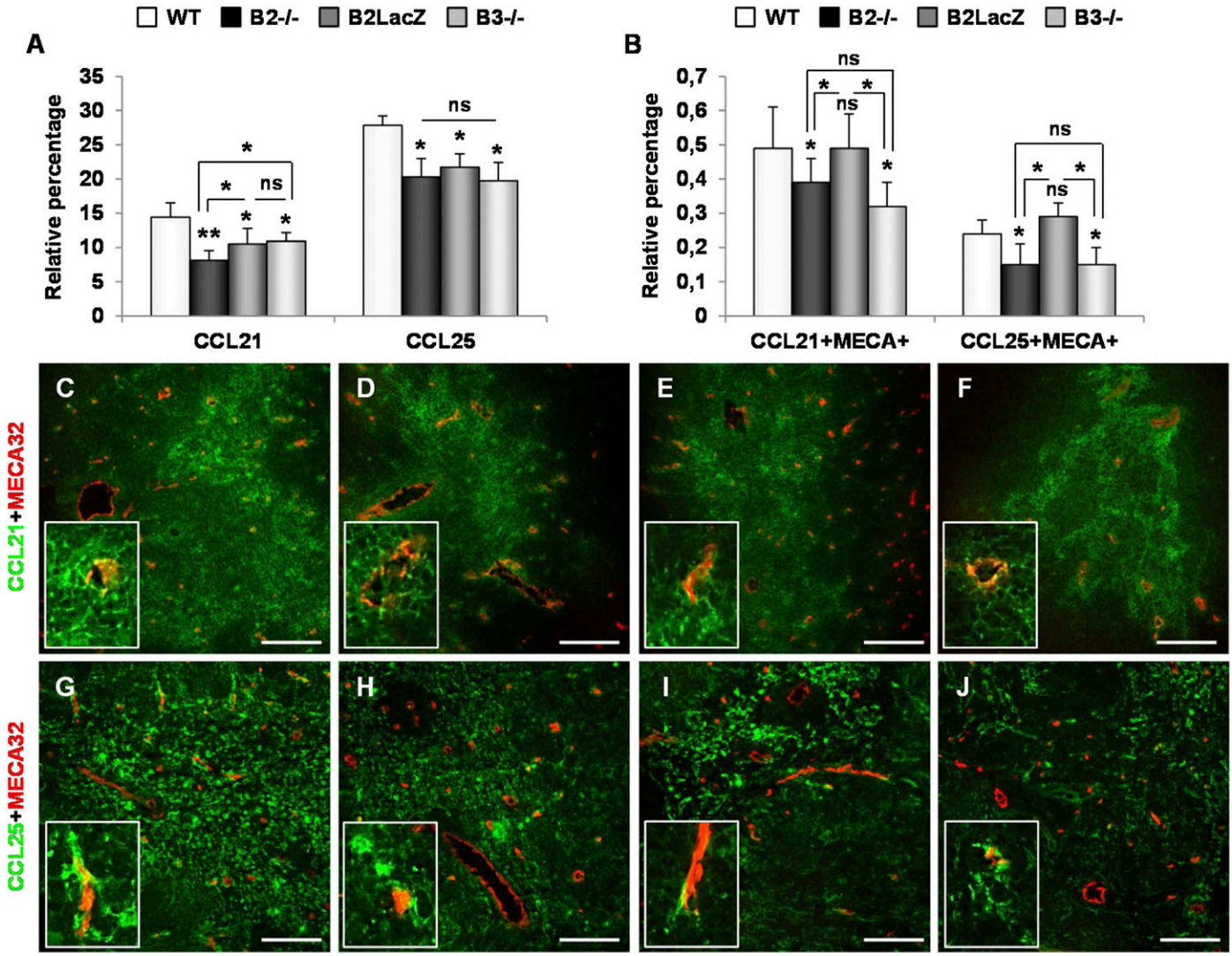

Figure 8. Chemokines involved in progenitor cell recruitment into the thymus are less expressed in EphB KO thymuses. (A) Data obtained from the analysis of successive thymic cryosections stained for either CCL21 or CCL25 showed lower expression of both molecules throughout the thymic stroma in all EphB-mutants studied compared with the WT thymuses. (B) When this analysis only referred to chemokine expression at the MECA32^{+} vascular endothelia, the expression of both CCL21 and CCL25 did not exhibit differences in EphB2LacZ thymuses compared with the WT thymuses but decreased significantly in both EphB2- and EphB3-deficient mice. (C–F) Representative thymic sections showing CCL21 expression (green) and MECA32 endothelial cells (red) in WT (C), EphB2$^{-/-}$ (D), EphB2LacZ (E), and EphB3$^{-/-}$ (F) thymuses. (G–J) Representative thymic sections showing CCL25 expression (green) and MECA32 endothelial cells (red) in WT (G), EphB2$^{-/-}$ (H), EphB2LacZ (I), and EphB3$^{-/-}$ (J) thymuses. In each case, inserts show the expression of CCL21 or CCL25 associated with MECA32^{+} blood vessels. *P* values from Student's *t* test statistical analysis are indicated as NS ($P \geq 0.05$), *$P < 0.05$, and **$P < 0.01$. Scale bars, 100 μm.

(HSCs, MPPs, ELPs, and CLPs) compared with WT mice. In contrast, EphB2LacZ mice only showed a diminished frequency of the most primitive stages, HSCs and MPPs. Thus, differences between the WT and mutant hematopoietic cell subsets gradually decreased in the more mature stages, confirming our previous results [13]. No changes were found in the proportions of EphB3$^{-/-}$ hematopoietic progenitor cells. As discussed later in the present report, the phenotypes of EphB2- and EphB3-deficient mice are not similar, with EphB2 KO mice exhibiting the most severe alterations. However, how the different hematopoietic progenitor cells contribute to thymic cell recruitment is a matter for discussion. As described, only hematopoietic progenitors that express CCR7, CCR9, and PSGL1 seem to be capable of efficiently colonizing the adult thymus [6, 8]. The proportion of these cells in the different BM cell subsets studied did not undergo significant changes when WT and mutant mice were compared. Nevertheless, other studies have reported that more primitive cells than CLPs, such as HSCs [44], lymphoid-primed multipotent progenitor (including in the subset analyzed in our study as MPPs plus ELPs) [45], or MPPs [31] retain the capacity to colonize the thymus. Because these studies have used different cell markers to identify the hematopoietic precursor subsets, the precise identity of the cells arriving into the thymus remains controversial. Presumably, all the progenitors identified in the adult BM have T-cell development potential, but only some, more restricted, subsets move from the peripheral

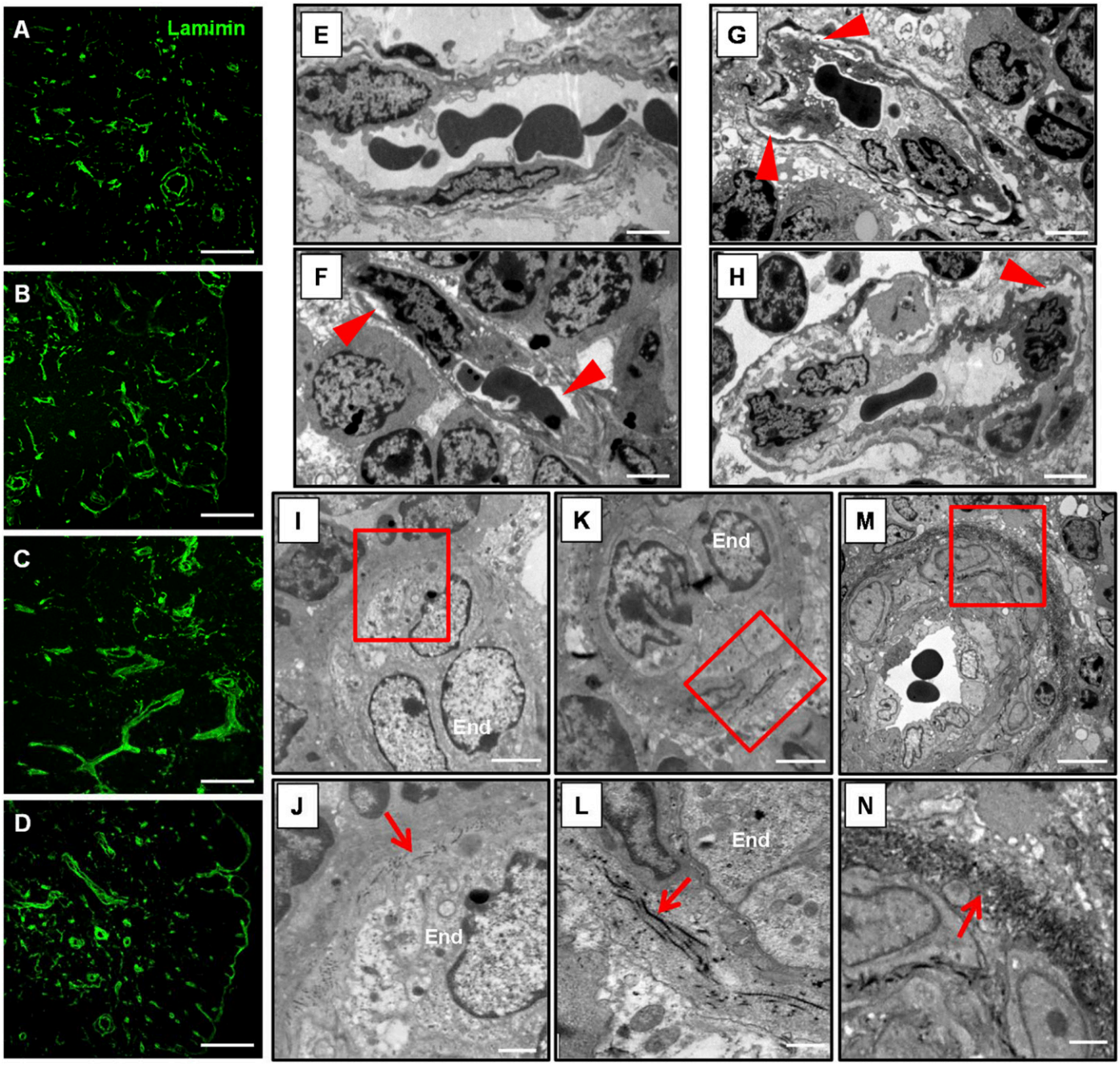

Figure 9. EphB-deficient mice showed morphologic changes in thymic blood vessels. (A–D) Blood vessels were identified by laminin expression. Note the increased staining occurring in EphB2$^{-/-}$ (B), EphB2LacZ (C), EphB3$^{-/-}$ (D) thymuses compared with WT ones (A). Scale bars, 200 μm. (E–H) Blood vessels of the thymic cortex examined by electron microscopy exhibited empty and enlarged spaces (arrowheads) between endothelial cells and surrounding cells, forming the vascular walls in EphB2$^{-/-}$ (F), EphB2LacZ (G), and EphB3$^{-/-}$ (H) compared with the condition of the WT cortical capillaries (E). Scale bars, 2 μm. (I–N) High endothelial venules (HEV) of EphB2$^{-/-}$ (K), EphB2LacZ (M) appear reinforced by enlarged layers of connective tissue (squares), showed (arrows) at higher magnifications in parts (L) and (N), respectively, compared with WT HEV (I and J). End, endothelial cells. Scale bars, 3 μm (I and K), 5 μm (M), and 1 μm (J, L, and N).

blood and enter the thymus [1]. Accordingly, decreased proportions of HSCs and MPPs found in both EphB2$^{-/-}$ and EphB2LacZ mice could also be contributing to the reduced migration into the thymus observed in these mutants.

Apart from the numbers of presumptive migrating cells from the BM to the thymus, it is known that different factors, including G-CSF, β-adrenergic agonists, and CXCR4 antagonists, modulate the exit of blood cell progenitors to the peripheral blood [46]. Some data have indicated that Eph/ephrins could also be involved in these processes. We have observed that human mesenchymal stem cells, a component of BM stroma, strongly express EphB2, EphB4, ephrinB1, and ephrinB2 and a short

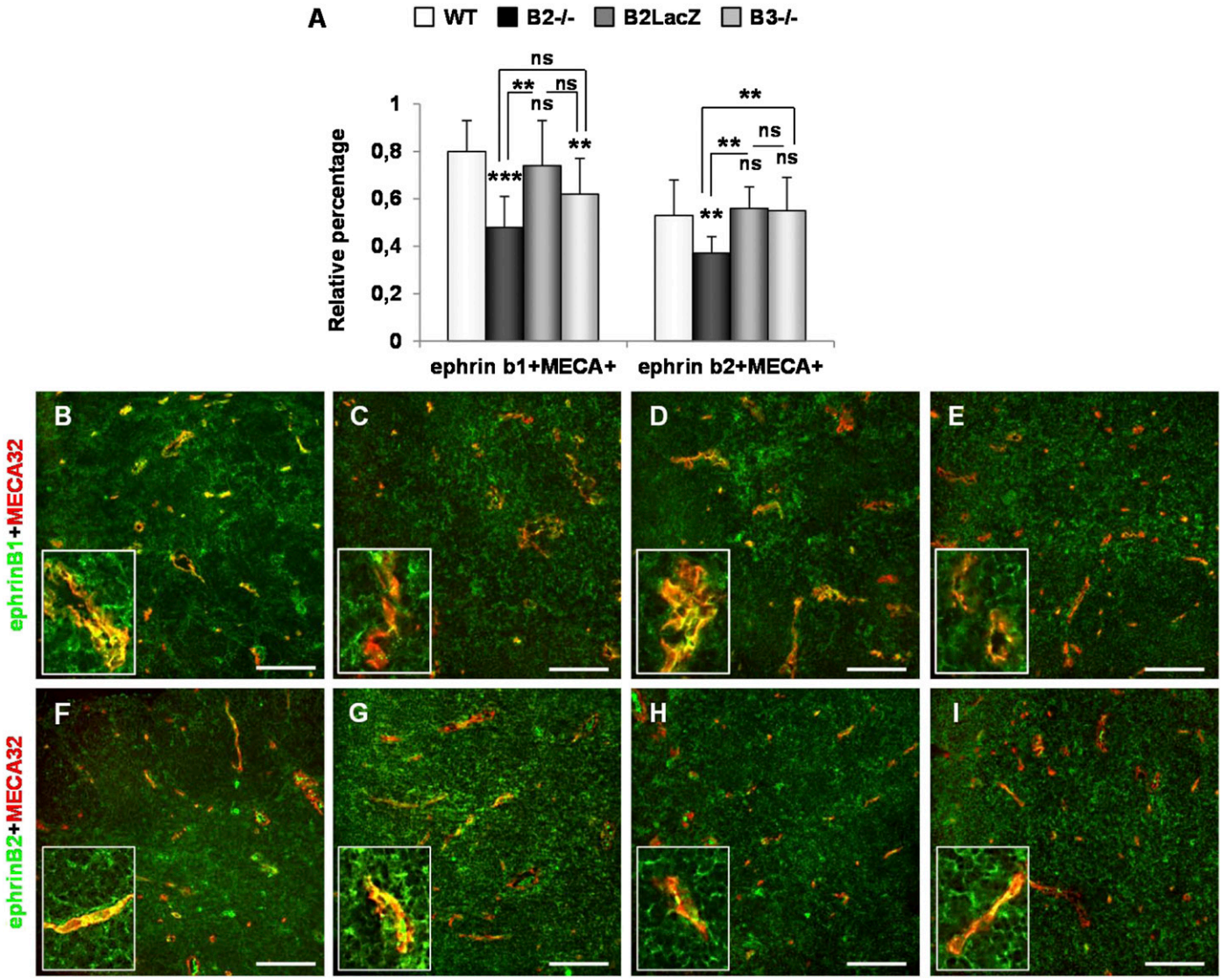

Figure 10. Expression of ephrinB1 and ephrinB2 in thymic blood vessels changes in EphB-deficient thymuses. (A) Lower expression of ephrinB1 in the thymic vasculature of both EphB2$^{-/-}$ and EphB3$^{-/-}$ mice but not in EphB2LacZ mice. In the case of ephrinB2, this reduction was only significant in EphB2-deficient mice; other mutants had values similar to those in WT. (B–E) Representative thymic sections showing ephrinB1 expression (green) and MECA32 endothelial cells (red) in WT (B), EphB2$^{-/-}$ (C), EphB2LacZ (D), and EphB3$^{-/-}$ (E) thymuses. (F–I) Representative thymic sections showing ephrinB2 expression (green) and MECA32 endothelial cells (red) in WT (F), EphB2$^{-/-}$ (G), EphB2LacZ (H), and EphB3$^{-/-}$ (I) thymuses. In each case, inserts show the expression of ephrinB1 or ephrinB2 associated with MECA32^{+} blood vessels. *P* values from Student's *t* test statistical analysis are indicated as NS ($P \geq 0.05$), $^{**}P < 0.01$, and $^{***}P < 0.005$. Scale bars, 100 μm.

EphA3 isoform and ephrinA4 (data not shown), confirming previous results [47–49]. In addition, sorted murine Lin^{-}Sca-1^{+}c-Kit^{+} (LSK) cells express EphA2 and EphA3 [50], and murine and human long-term HSCs express ephrinB2 [51]. Although this was not analyzed in the present study, Eph–ephrin interactions between hematopoietic progenitor cells and stromal components could be involved in the mobilization of the former, and alterations in mutant mice would contribute to changes in migration toward the thymus. Thus, EphB4^{+} hematopoietic precursor cell migration within the BM is inhibited by ephrinB2^{+} stromal cells. In addition, BM Stro-1^{+} mesenchymal stem cells stimulated by ephrinB1-Fc proteins show increased in vitro migration and EphB2 silencing alters the migratory properties [47]. Also, EphA3-Fc protein treatment mobilizes primitive hematopoietic progenitors in the peripheral blood [50]. In the BM, just as in other systems, Eph-mediated cell mobilization could be mediated through CXCL12. Ephs interact with CXCR4, the CXCL12 receptor, on the cell surface [52], the ephrinB1-Fc fusion protein stimulates the CXCL12-dependent migration of peripheral blood lymphocytes in a dose-dependent manner [26] and Eph–ephrin interactions inhibit the CXCL12-mediated chemotaxis of cerebellar granular cells [53].

The thymic colonization performed by EphB-deficient progenitors in WT adult thymuses and that of WT precursors

into EphB-deficient mice follows different rules, the comparative analysis of which provides interesting information on the regulatory mechanisms exerted by EphB in the lymphoid precursor colonization of the adult thymus. Mutant BM progenitor cells show a decreased capacity to colonize WT thymuses (Fig. 4A), and this finding was more evident when mutant cells were injected competitively, together with WT Lin^- BM cells (Fig. 4B). Such a phenomenon was previously observed in other models deficient in homing molecules, such as CCR7/CCR9 double KO mice [30]. In the 3 conditions studied, progenitor recruitment was importantly reduced, most severely in the case of $EphB2^{-/-}$ progenitors. The lack of EphB2 forward signaling and, to a lesser degree, that of EphB3 forward signaling are determinant to explain these results, indicating the relevance of EphB2, rather than EphB3, forward signals for progenitor cell recruitment to adult thymus. In the case of EphB2LacZ, the occurrence of a reverse signal presumably transmitted to thymic endothelial cells improved, but did not totally recover, colonization to WT levels. Remarkably, our previous studies using an in vitro reconstituting assay in fetal thymic lobes demonstrated that in these experimental conditions the reverse signal transmitted by ephrinB expressed in fetal thymic stroma was sufficient for recovering thymic progenitor settling [13]. These results, therefore, suggest that reverse signaling exerted on endothelial cells favors the lymphoid settling to thymus and confirm that fetal and adult thymic colonization (and in vivo and in vitro assays) are not equivalent, as previously reported [2].

WT Lin^- BM cells, assayed to in vivo colonize mutant thymuses, also showed decreased seeding capacity, particularly significant in EphB2-deficient thymus (Fig. 5). This finding demonstrates the relevance of the thymic microenvironment in governing precursor cell recruitment, supporting other previous results [6, 35]. In these assays, the expression of EphB2LacZ in the thymic cells also provides important improvement in WT progenitor cell recruitment, although the differences remain statistically significant compared with the WT thymuses. In this case, the lack of EphB forward signals in thymic endothelial cells could explain the reduced colonization of the thymus. In contrast, reverse signals transmitted to ephrinB-expressing progenitor cells improved, but did not totally rescue, the colonization capacity of WT BM cells. Therefore, forward signaling in either progenitor cells or thymic stroma is key for in vivo lymphoid recruitment into the thymus. In contrast, ephrinB reverse signaling appears to be secondary. However, the reduction in intrathymic migrating capacity is more severe when mutant cells are injected in WT thymuses than when mutant mice receive WT BM progenitor cells. These data suggest that the lack of forward signals in circulating progenitor cells is more important that their absence in the thymic stroma.

Our studies on the colonization capacity of EphB-deficient Lin^- BM cells to WT or EphB-deficient thymuses (Fig. 6) confirmed our conclusions on the relevance of distinct Eph/ephrinB signals for the process. $EphB2^{-/-}$ progenitor cells exhibited the lowest colonization to any tested thymus and were particularly low when injected into EphB2-deficient mice. In this condition, both EphB2 forward and reverse signaling were abrogated in the progenitor cells and thymic endothelia. Any mutant progenitor cell exhibits the lowest colonization capacity injected in EphB2 KO mice. Both findings have confirmed the relevance of EphB2 expressed in both progenitors and thymic endothelial cells for lymphoid recruitment to the thymus. EphB3 and EphB2LacZ thymuses showed reduced colonization but significantly greater than that found in $EphB2^{-/-}$ mice. In addition, both types of deficient progenitors colonized EphB2LacZ and EphB3 mice similarly. However, it is especially remarkable that the lack of EphB3 receptors, which results in defective signaling quite similar to that caused by the absence of EphB2 [54], courses with a significantly better capacity for progenitor cell seeding to the thymus, confirming its negligible involvement in the process. As indicated, EphB2- and EphB3-deficient thymuses are not equal, because, in general, the phenotype observed in $EphB2^{-/-}$ mice is more severe [17]. In addition, a signaling imbalance between these 2 molecules induces different phenotypes [18]. Chimaeras established with $EphB2^{-/-}$ progenitor cells injected in SCID mice showed an almost total blockade of thymocyte development at the double negative stage. However, in similar conditions, $EphB3^{-/-}$ generated double-positive cells, although at low proportions [18]. Although the pathways activated by these molecules are, in general, common, the system exhibits a certain specificity dependent on the cell type, the context of cell stimulation, and interactions with other signaling systems [54].

In contrast, when reverse signaling is induced by EphB2LacZ progenitor cells in $EphB2^{-/-}$ thymic endothelial cells, a mild recovery of colonization occurs that is slightly greater when the signal occurs in $EphB3^{-/-}$ thymic cells. Even with the occurrence of reverse signaling in both progenitor and thymic cells (EphB2LacZ precursors in EphB2LacZ thymus), the recovery did not reach WT levels. These results have confirmed the importance of forward vs. reverse signaling in controlling the lymphoid seeding to the thymus.

Together, these results suggest that, if the frequencies of WT and mutant migrating cells, mainly those expressing CCR7, CCR9, and/or PSGL1, to the thymus do not show significant differences, the processes associated with chemotaxis and migration throughout the thymic blood vessels will become particularly important for explaining the present results. This idea has been further reinforced by the finding that Lin^- WT progenitors migrated less efficiently to the mutant thymuses than did those colonizing WT ones. The recruitment of circulating cells is controlled by the combined action of selectins, integrins, and adhesion molecules of the Ig superfamily in concert with chemokine gradients [55]. Numerous data support the relationships among Eph, chemokine/chemokine receptors, and cell migration in different experimental models, including the immune system, although the results are controversial. EphA2 and its ligand ephrinA4 appear to be involved in B cell trafficking [56], and the absence of EphA2 alters the extravasation of immunocompetent cells [22, 27]. EphB-expressing monocytes adhere preferentially to, and transmigrate through, ephrinB2-expressing endothelia [55], and activation of endothelial ephrinB2 reverse signaling has proadhesive and promigrating effects on endothelial cells. In contrast, EphB4 signaling interferes negatively [57]. EphrinB1-Fc proteins stimulate the CXCL12-dependent migration of peripheral blood lymphocytes

[26], and ephrinA1, combined with CXCL12 or CCL19, stimulates migration of memory T cells [58], but ephrinA1, A3, and B2 inhibit CXCL12-induced chemotaxis of thymocytes [27].

In the thymuses of EphB-deficient mice we studied, our present results have demonstrated, as previously reported [37], altered morphologic organization of blood vessels in mutant thymuses, evaluated by laminin expression and electron microscopy examination, and a significant decrease in CCL21 and CCL25 chemokine expression compared with that found in WT thymuses. This finding confirms our previous data on the EphB2$^{-/-}$ and EphB2LacZ thymuses [13]. As indicated, CCR7 and CCR9 receptors have been implicated in thymic homing in fetal and adult mice [4, 8, 30, 59]. Presumably, other chemokine receptors are also involved in progenitor migration into the thymus (e.g., CXCR4 and CCR5), but their contribution requires additional confirmation [60]. Our results have also demonstrated CCL21 expression in the thymic MECA32^{+} blood vessel endothelia that could be key to the interaction with CCR7^{+} circulating progenitor cells and favor their migration, just as other investigators have emphasized [1]. A quantitative study of this CCL21 expression associated with thymic endothelia again showed a significant decrease in mutant thymuses with respect to WT values. In contrast, CCL25 is strongly expressed by TECs throughout the thymic parenchyma and only appears to be slightly associated with some blood vessels. Thus, presumably, a gradient of CCL25 molecules would permeate the blood vessel walls to participate in the attraction of circulating CCR9^{+} migrating cells.

Both CCL21 and CCL25 expression was significantly diminished in thymic MECA32^{+} endothelial cells in both EphB2$^{-/-}$ and EphB3$^{-/-}$ mice but not in EphB2LacZ mice compared with WT thymuses. Likewise, the reduction in P-selectin expression is very important in both EphB2- and EphB3-deficient thymuses but does not show significant differences to WT values in the EphB2LacZ thymuses. In agreement, we had demonstrated previously that the migration mediated by chemokines of EphB2$^{-/-}$ Lin^{-} BM-derived progenitor cells, examined in transwell assays, was significantly reduced [13]. Next, we hypothesized that the lack of chemokine receptor costimulation by EphB2 in EphB2$^{-/-}$ progenitor cells could account for the reduced migration, but that in EphB2LacZ progenitors, the presence of the extracellular domain could evoke certain cross-stimulations, which might explain the greater degree of thymic colonization found in these mice. Crosstalk among Eph/ephrins, integrins, and chemokine receptors has been repeatedly described in other systems, concluding the participation of different intracellular pathways that modulate activation of second messengers, including Ras, Rho, focal adhesion kinase, and others [61].

The relationships between Eph/ephrins and selectins have been less studied but EphB4 activation upregulates PSGL1 in endothelial progenitor cells, resulting in increased homing [62]. In contrast, although the absence of EphB2 or EphB3 from the cell surface of lymphoid progenitors did not seem to affect their PSGL1 expression, decreased expression of its ligand, P-selectin, on the thymic endothelial cells of both EphB2$^{-/-}$ and EphB3$^{-/-}$ mice could contribute to the reduced thymic colonization observed in those mice.

Changes in the expression of ephrinB studied in EphB-deficient thymuses are also relevant for explaining the decreased migration into the thymus of EphB-deficient mice. Again, no significant differences were found in the expression of both ephrinB1 and ephrinB2 on the endothelia of EphB2LacZ thymuses. However, the expression of these ephrinB decreases significantly in the vascular endothelia of EphB2-deficient thymuses, more severely in the case of ephrinB1. EphrinB1 expression is also reduced in the EphB3$^{-/-}$ thymic endothelia compared with that of WT ones. However, no significant differences were seen in the expression of ephrinB2. Together, these results suggest that ephrinB1 could be more relevant than ephrinB2 for lymphoid seeding of the adult thymus. On this respect, in a cancer cell line, EphB2 is strongly phosphorylated by ephrinB1, weakly by ephrinB2, and little or not at all by ephrinB3 [63]. Thus, our results show an interesting correlation among reduced lymphoid colonization of the adult thymus, the absence of either EphB2 or EphB3, and changes in the thymus microenvironment that affects the expression of certain chemokines, P-selectin, and EphB ligands. Moreover, the forward signals mediated by the EphB2-ephrinB1 pair signaling appear to be the most important regulatory factor of the process.

AUTHORSHIP

D.A. conceived, designed, and performed the experiments, analyzed the data, and wrote the report; J.G.-C. assisted in colonization assays, electron microscopy studies, and data analysis; D.F.-O. contributed to the colonization assays and data analysis; E.T.-G. contributed to the FACS analysis; S.M. assisted in animal mating and genotyping; V.C.-A. contributed to the data analysis and revised the manuscript; W.S. contributed to the data analysis and revised the manuscript; A.Z. contributed to the experiment design, electron microscopy studies, data analysis, reagents, and wrote the report.

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DISCLOSURES

The authors declare no competing financial interests.

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