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Inhibition of CD10/neutral endopeptidase 24.11 promotes B-cell reconstitution and maturation *in vivo*

(common acute lymphoblastic leukemia antigen/lymphoid progenitors/differentiation/cell surface enzyme)

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ABSTRACT **The common acute lymphoblastic leukemia antigen [(CALLA) CD10, neutral endopeptidase 24.11 (NEP)] is a cell-surface zinc metalloprotease expressed by a subpopulation of early murine B-lymphoid progenitors and by bone marrow stromal cells that support the earliest stages of B lymphopoiesis. In previous *in vitro* studies in which uncommitted murine hematopoietic progenitors plated on a stromal cell layer differentiate into immature B cells, the inhibition of CD10/NEP increased early lymphoid colony numbers. To further characterize CD10/NEP function during lymphoid ontogeny *in vivo*, we utilized a Ly5 congenic mouse model in which the lymphoid differentiation of uncommitted hematopoietic progenitors from Ly5.1 donors was followed in sublethally irradiated Ly5.2 recipients treated with a specific long-acting CD10/NEP inhibitor {*N*-[L-(1-carboxy-2-phenyl)ethyl]-L-phenylalanyl-β-alanine (SCH32615)}. The expression of Ly5.1, B220, and surface IgM (sIgM) was utilized to characterize donor-derived hematopoietic cells (Ly5.1$^+$), B lymphocytes (B220$^+$), and mature B cells (B220$^+$ sIgM$^+$) from the lymphoid organs of recipient animals treated with SCH32615 or vehicle alone. SCH32615-treated animals had higher percentages of Ly5.1$^+$ donor splenocytes than animals treated with vehicle alone (16.9% vs. 10.4%, 63% increase, $P = 0.013$). Animals treated with the CD10/NEP inhibitor also had relatively more Ly5.1$^+$ splenic B (B220$^+$) cells than vehicle-treated animals (14.4% vs. 8.2%, 75% increase, $P = 0.018$). To more specifically characterize the effects of CD10/NEP inhibition on B-cell differentiation, Ly5.1$^+$ splenocytes from animals treated with SCH32615 or vehicle alone were analyzed for coexpression of B220 and sIgM. Animals treated with the CD10/NEP inhibitor had a significantly higher percentage of mature donor B cells (Ly5.1$^+$ B220$^+$ sIgM$^+$, 10.2% vs. 5.2%, 90% increase, $P = 0.006$) and a more modest relative increase in immature donor B cells (Ly5.1$^+$ B220$^+$ sIgM$^-$, 4.7% vs. 3.4%, 38% increase, P = not significant). Taken together, these results suggest that CD10/NEP inhibition promotes the reconstitution and maturation of splenic B cells. Therefore, CD10/NEP may function to regulate B-cell ontogeny *in vivo* by hydrolyzing a peptide substrate that stimulates B-cell proliferation and/or differentiation.**

The common acute lymphoblastic leukemia antigen (CALLA, CD10) is a 100-kDa cell-surface glycoprotein expressed by the majority of acute lymphoblastic leukemias and by other lymphoid malignancies with an immature phenotype (1, 2). Normal lymphoid progenitors that are either uncommitted or committed to only the earliest stages of B- or T-cell differentiation also express CD10 (3–6), suggesting that the protein plays a role in the early stages of lymphoid ontogeny. However, CD10 is also expressed on granulocytes and nonhematopoietic cell types including bronchial epithelial cells, cultured fibroblasts, renal proximal tubular epithelial cells, and certain solid tumor cell lines, indicating that its biologic function is not restricted to lymphoid development (7–9).

The molecular and functional characterization of CD10 demonstrated it to be identical to the cell-surface zinc metalloprotease neutral endopeptidase 24.11 (NEP) (10–12). CD10/NEP cleaves peptide bonds on the amino side of hydrophobic amino acids and inactivates a variety of peptide hormones including enkephalin, chemotactic peptide [formyl-Met-Leu-Phe (FMLP)], substance P, atrial natriuretic factor, endothelin, oxytocin, neurotensin, bradykinin, angiotensin, and the bombesin-like peptides (13–18).

In the cell types in which it has been studied, the enzyme down-regulates cellular responses to peptide hormones. For example, CD10/NEP down-regulates substance P-mediated neurogenic inflammation of the lung (15, 19, 20), atrial natriuretic factor-mediated blood pressure control and diuresis (16), and [Met]enkephalin- and FMLP-induced neutrophil inflammatory responses (14, 21). Target cells express CD10/NEP and the receptor for the relevant CD10/NEP peptide substrate. By hydrolyzing the peptide, CD10/NEP reduces local concentrations of peptide available for receptor binding and signal transduction.

Recent studies indicate that CD10/NEP also regulates peptide-mediated cellular proliferation (17, 22). The enzyme hydrolyzes bombesin-like peptides, which are potent mitogens for fibroblasts, normal bronchial epithelial cells, and certain small cell carcinomas of the lung (17). The proliferation of bombesin-like peptide-dependent small cell carcinomas is inhibited by CD10/NEP and potentiated by CD10/NEP inhibition (17). The bombesin-like peptide-dependent growth of normal fetal lung is similarly modulated by the cell-surface enzyme (22).

Although CD10/NEP has a known role in many organ systems, until recently little was known about the enzyme's function in lymphoid ontogeny. The absence of suitable human models for the study of lymphoid CD10/NEP prompted us to turn to the murine system in which discrete stages of B-cell development and models of *in vitro* and *in vivo* lymphoid differentiation are well characterized (23, 24). We cloned the murine CD10/NEP cDNA, which predicts a 750-amino acid type II integral membrane protein with 90% identity to the human CD10/NEP sequence and 100% conservation of critical amino acids and functional motifs (25).

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Abbreviations: NEP, neutral endopeptidase 24.11; CALLA, common acute lymphoblastic leukemia antigen; sIgM, surface IgM.

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CD10/NEP transcripts and enzymatic activity were primarily restricted to a subpopulation of murine lymphoid progenitors isolated from bone marrow and modified Whitlock–Witte bone marrow cultures that coexpressed B220 and low levels of Thy-1 (26). These Thy-1^{low} B220^{+} cells are large cycling cells that can effectively reconstitute the B-lymphoid system of lethally irradiated animals (27, 28). Bone marrow stromal cells known to support the B-cell differentiation of hematopoietic progenitors including Thy-1^{low} B220^{+} cells also expressed CD10/NEP (26). For these reasons, we evaluated the effects of specific CD10/NEP inhibitors in modified Whitlock–Witte cultures of uncommitted hematopoietic progenitors plated on a stromal cell line that supports the earliest stages of B-cell differentiation. In such an *in vitro* system in which uncommitted Thy-1^{low} Lin^{-} hematopoietic progenitors differentiate into Thy-1^{low} B220^{+} and Thy-1^{-} B220^{+} B-cell precursors, CD10/NEP inhibition increased the numbers of early lymphoid colonies (26).

To further characterize CD10/NEP function *in vivo*, we have utilized an Ly5 congenic mouse model in which the lymphoid differentiation of uncommitted hematopoietic progenitors from Ly5.1 donors can be followed in sublethally irradiated Ly5.2 recipients treated with a long-acting inhibitor of CD10/NEP {*N*-[L-(1-carboxy-2-phenyl)ethyl]-L-phenylalanyl-β-alanine (SCH32615)}. SCH32615 has been shown in previous studies to specifically inhibit CD10/NEP enzymatic activity and to increase CD10/NEP substrate-mediated physiological effects on other CD10/NEP^{+} cell types (29–31).

MATERIALS AND METHODS

Congenic Mice. C57BL/6 (Ly5.1) mice were obtained from The Jackson Laboratory and B6Ly5.2 mice were provided by Clarence Reeder (NCI, Frederick, MD). Mice were maintained at the Redstone Animal Facility (Dana–Farber Cancer Institute, Boston).

CD10/NEP Inhibition. The specific CD10/NEP competitive inhibitor SCH32615 (Schering–Plough) (29–31) was provided by Jeffrey Drazen (Brigham & Women's Hospital, Boston). SCH32615 was dissolved in dimethyl sulfoxide and stored at 4°C for up to 5 days at a concentration of 10 mg/ml. Immediately prior to its use, SCH32615 was diluted to the appropriate concentration (0.1 mg/ml) in phosphate-buffered saline (PBS) and administered at a dose of 1 mg/kg i.p. twice daily. This dose and route of administration were chosen because they were effective in inhibiting systemic CD10/NEP enzymatic activity and increasing a variety of CD10/NEP substrate-mediated physiological effects in previous murine *in vivo* studies (29, 32).

B-Cell Reconstitution in Congenic Animals Treated with a CD10/NEP Inhibitor. The congenic Ly5.1 and Ly5.2 mice strains differed only in their expression of allelic forms of the CD45 family member, Ly5 (33). In each of several experiments, 8- to 10-week-old Ly5.2 littermates were divided into two treatment groups. On day −2, one group of animals began treatment with SCH32615, 1 mg/kg i.p. twice daily. The other group began treatment with an identical concentration of dimethyl sulfoxide diluted in PBS (vehicle alone) (Fig. 1). On day 0, Thy-1^{low} Lin^{-} uncommitted hematopoietic progenitors were isolated from the bone marrow of 3-week-old C57BL/6 Ly5.1 donor animals as described (26). On day 0, Ly5.2 animals were treated with 800 rads (1 rad = 0.01 Gy) delivered in two equal fractions separated by at least 6 hr. Radiation was administered using a ^{60}Co γ irradiation source (Atomic Energy of Canada, Ottawa). After the radiation was completed, 10^4 Thy-1^{low} Lin^{-} bone marrow donor cells from Ly5.1 mice were injected into the tail vein of each Ly5.2 recipient animal (Fig. 1). Ly5.2 recipient animals were then treated for an additional 21 days with either SCH32615 or vehicle alone. On day 21, spleens and lymph nodes from Ly5.2 recipient animals were harvested and cell suspensions from these organs were analyzed for Ly5.1, B220, surface IgM (sIgM), and Thy-1 expression (Fig. 1). Phenotypic analyses were performed on day 21 because pilot experiments indicated that this was the first time point at which significant numbers of Ly5.1^{+} cells could be identified in the spleens and lymph nodes of Ly5.2 animals.

Antibodies. Unconjugated first-step antibodies utilized in this study included rat anti-B220, anti-Mac-1, anti-Gr-1, anti-Ly2/3, and appropriate negative controls (26). Directly conjugated first-step antibodies utilized in the experiments included rat anti-mouse-IgM-biotin (PharMingen), rat anti-Thy-1-biotin (Becton Dickinson), rat anti-B220-phycoerythrin (PE) (Caltag, South San Francisco, CA), mouse anti-Ly5.1-fluorescein isothiocyanate (FITC) (34), and isotype-matched controls (IgG2b-biotin and IgG2a-PE; Caltag). Second-step reagents included goat anti-rat-PE (Tago), streptavidin-FITC (Tago), and streptavidin-Red 613 (GIBCO/BRL).

Immunofluorescence Staining and Analysis. Thy-1^{low} Lin^{-} (B220^{-} Mac-1^{-} Gr-1^{-} Ly2/3^{-}) bone marrow uncommitted hematopoietic progenitors (35) from C57BL/6 Ly5.1 animals were phenotyped and isolated as described (26).

Single cell suspensions from the whole spleens and lymph nodes of recipient animals were phenotyped by serially incubating cells with (*i*) biotinylated anti-Thy-1 or anti-IgM, (*ii*) B220-PE, (*iii*) avidin-Red 613, and (*iv*) Ly5.1-FITC as described (26, 34). All incubations were performed in the presence of 5% normal rat serum (Pierce). Immunofluorescence analyses were performed on a FACScan (Becton Dickinson). In these analyses, 2×10^4 (lymph nodes) or 3×10^4 (spleen) events were collected, gating for viable lymphoid cells by forward scatter and side scatter fluorescence. Fluorescence intensity was displayed on a logarithmic scale and contour plots were generated using LYSYS software (Becton Dickinson). The percentage of cells staining with a given antibody combination was determined using positive and negative quadrants or logical gates (see *Results and Discussion*) and the results in SCH32615-treated and control groups were compared using a one-sided Student *t* test.

RESULTS AND DISCUSSION

Analysis of B-Cell Reconstitution in Congenic Animals. To analyze the effects of CD10/NEP inhibition on *in vivo* B-cell proliferation and differentiation, Ly5.1^{+} donor Thy-1^{low} Lin^{-} uncommitted hematopoietic progenitors were injected into sublethally irradiated Ly5.2 recipient animals pretreated with either SCH32615 or vehicle alone (Fig. 1). After an additional 21 days of treatment, the lymphoid reconstitution in the spleen and lymph nodes of SCH32615- and vehicle-treated animals was further assessed (Fig. 1). The expression of Ly5.1, B220, and sIgM was utilized to characterize donor-derived hematopoietic cells (Ly5.1), B lymphocytes (B220), and mature B cells (B220, sIgM) from the spleens and lymph nodes of recipient animals.

Examples of Ly5.1, B220, and sIgM triple-color staining of splenocytes from recipient animals treated with SCH32615 or vehicle alone are shown in Fig. 2 *B*, *C*, *E*, and *F*. For comparison, the Ly5.1/B220 staining of splenocytes from an Ly5.2 animal reconstituted with syngenic (Ly5.2) bone marrow is shown in Fig. 2 *A* and *D*. As expected, no Ly5.1^{+} cells were present in the Ly5.2 animal reconstituted with syngenic bone marrow (Fig. 2*A*). In contrast, substantial numbers of Ly5.1^{+} splenocytes were detected in Ly5.2 recipients reconstituted with Thy-1^{low} Lin^{-} uncommitted hematopoietic progenitors from Ly5.1 donor animals (Fig. 2 *B* and *C*). The majority of these donor-derived Ly5.1^{+} splenocytes were B220^{+} B cells (Fig. 2 *B* and *C*). To further characterize the

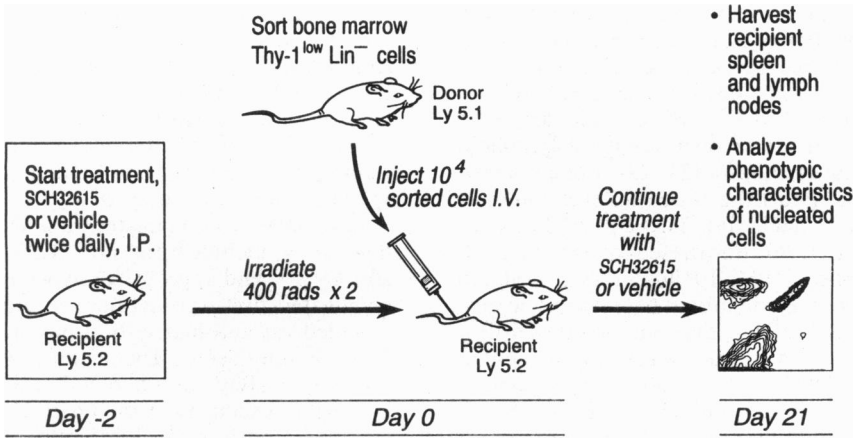

FIG. 1. Lymphoid reconstitution in congenic animals treated with a CD10/NEP inhibitor: Schema. On day −2 recipient B6Ly5.2 mice began to receive either SCH32615, 1 mg/kg per injection, or vehicle alone twice daily i.p. On day 0, recipient Ly5.2 mice were sublethally irradiated (400 rads × 2 at 6-hr intervals). On the same day, bone marrow was harvested from 3-week-old C57BL/6-Ly5.1 animals, and Thy-1^{low} Lin$^-$ uncommitted hematopoietic progenitors were identified by immunofluorescence and sorted as described (26). After irradiation was completed, 10^4 Thy-1^{low} Lin$^-$ bone marrow donor cells were injected into the tail veins of each irradiated Ly5.2 recipient animal. Ly5.2 recipient animals were then treated for an additional 21 days with either SCH32615 or vehicle alone. On day 21, spleens and lymph nodes from Ly5.2 animals were harvested and analyzed for Ly5.1, B220, sIgM, and Thy-1 expression. The histogram shown is of splenocytes analyzed for Ly5.1 (*x* axis) and B220 (*y* axis) (see Fig. 2).

stage of differentiation of these donor B cells, the Ly5.1$^+$ cells were gated as shown (Fig. 2 *B* and *C*) and analyzed for the coexpression of B220 and sIgM (Fig. 2 *E* and *F*). For each syngenic or congenic animal, the percentages of all splenic cells expressing B220 and Ly5.1 (Fig. 2 *A–C*) or Ly5.1 and a combination of B220 and sIgM (Fig. 2 *D–F*) were then determined.

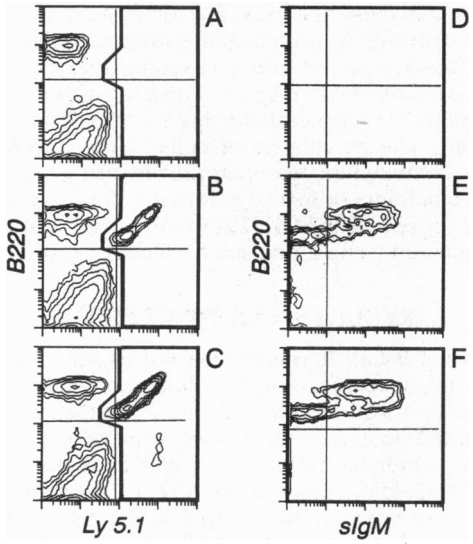

FIG. 2. Phenotypic analysis of splenic cells from Ly5.2 recipients reconstituted with syngenic (Ly5.2$^+$) or congenic (Ly5.1$^+$) donor hematopoietic progenitors. (*A–C*) Ly5.1 (*x* axis) and B220 (*y* axis) immunofluorescence of splenic cells. (*A*) Ly5.2 recipient reconstituted with syngenic (Ly5.2) bone marrow cells following irradiation. (*B*) Vehicle-treated Ly5.2 recipients reconstituted with Ly5.1$^+$ Thy-1^{low} Lin$^-$ bone marrow cells. (*C*) SCH32615-treated Ly5.2 recipients reconstituted with Ly5.1$^+$ Thy-1^{low} Lin$^-$ bone marrow cells. In all animals, Ly5.1$^+$ splenocytes were gated as shown (enclosure) for additional analysis as described in *D–F*. (*D–F*) sIgM (*x* axis) and B220 (*y* axis) immunofluorescence of gated Ly5.1$^+$ splenic cells from the above-mentioned syngenic and vehicle- and SCH32615-treated congenic recipients. The Ly5.1$^+$ splenocytes from syngenic (*D*) and vehicle- (*E*) and SCH32615-treated (*F*) congenic animals were gated as shown above and analyzed for the coexpression of B220 and sIgM. As expected, the syngenic animal had no Ly5.1$^+$ donor cells to further characterize (*A* and *D*). In these representative animals, the percentages of all splenic cells expressing B220 and Ly5.1 (*A–C*) or Ly5.1 and a combination of B220 and sIgM (*D–F*) were calculated for further analysis (Tables 1–3).

CD10/NEP Inhibition Increases Donor-Derived B-Cell Reconstitution in Spleen. Table 1 summarizes a representative experiment in which the relative percentages of Ly5.1$^+$ donor splenocytes from groups of Ly5.2 animals treated with SCH32615 or vehicle alone are compared. SCH32615-treated animals had 63% more Ly5.1$^+$ donor splenocytes than animals treated with vehicle alone (16.9% vs. 10.4%, $P = 0.013$). Furthermore, animals treated with the CD10/NEP inhibitor had 75% more Ly5.1$^+$ splenic B (B220$^+$) cells than control animals (14.4% vs. 8.2%, $P = 0.018$; Table 1). Taken together, these results suggest that CD10/NEP inhibition is associated with more efficient donor B-cell reconstitution.

Although there were relatively increased numbers of donor-derived splenic B cells in SCH32615-treated animals, there were no significant differences in the numbers of

Table 1. CD10/NEP inhibition increases the percentages of donor-derived B cells in spleen

Origin	Lineage	% of cells		% change	*P* value
		Vehicle	SCH32615		
Donor					
Ly5.1$^+$	All	10.4 ± 3.4	16.9 ± 5.4	+63	0.013
Ly5.1$^+$	B220$^+$	8.2 ± 3.4	14.4 ± 5.5	+75	0.018
Ly5.1$^+$	B220$^-$	2.1 ± 0.6	2.5 ± 0.9	+19	NS
Recipient					
Ly5.1$^-$	B220$^+$	12.4 ± 5.7	13.7 ± 9.5	+10	NS

One of three representative experiments is shown, in which two groups of seven Ly5.2 animals each were treated with either SCH32615 or vehicle alone, irradiated, given Thy-1^{low} Lin$^-$ progenitors from Ly5.1 donors, and subsequently analyzed on day 21 as described in the legend to Fig. 1. One vehicle-treated animal died prior to the analysis. Cell suspensions from the whole spleen of each vehicle- and SCH32615-treated animal were characterized for the presence of donor-derived hematopoietic cells (Ly5.1$^+$) and B lymphocytes (B220$^+$) as described in the legend to Fig. 2. The percentages of spleen cells in each animal that were Ly5.1$^+$, Ly5.1$^+$ B220$^+$, Ly5.1$^+$ B220$^-$, and Ly5.1$^-$ B220$^+$ were determined as described (Fig. 2). For the two groups of vehicle- and SCH32615-treated animals, the mean percentages (±SD) of spleen cells expressing each phenotype were calculated and the resulting values were compared using a one-sided Student *t* test. NS, not significant.

endogenous splenic B cells (Ly5.1$^-$ B220$^+$) in animals treated with SCH32615 or vehicle alone (13.7% vs. 12.4%, Table 1). In the Ly5.2 recipients, the Ly5.1$^+$ donor splenocytes were derived from Thy-1^{low} Lin$^-$ uncommitted hematopoietic progenitors, whereas the endogenous splenocytes were derived from progenitors that survived sublethal irradiation at a variety of stages of hematopoietic differentiation. It is possible that the effect of CD10/NEP inhibition is most apparent when a relatively homogeneous population of uncommitted progenitors like the Ly5.1$^+$ Thy-1^{low} Lin$^-$ cells undergoes hematopoietic differentiation.

There were similar percentages of donor-derived non-B cells (Ly5.1$^+$ B220$^-$) in the spleens of vehicle- and SCH32615-treated animals (2.1% vs. 2.5%, Table 1). The numbers of donor-derived T cells (Ly5.1$^+$ B220$^-$ Thy-1^{bright}) in both types of animals were too low (<0.5%) to evaluate the effects of CD10/NEP inhibition on splenic T-cell reconstitution (data not shown).

CD10/NEP Inhibition Increases Donor-Derived B-Cell Maturation in Spleen. To more specifically characterize the effects of CD10/NEP on donor-derived B-cell differentiation, the Ly5.1$^+$ splenocytes from Ly5.2 animals treated with vehicle alone or SCH32615 were gated (as shown in Fig. 2 *B/C*) and analyzed for the coexpression of B220 and sIgM (as shown in Fig. 2 *D/E*). In the same representative experiment described in Table 1, animals treated with the CD10/NEP inhibitor had a 90% relative increase in mature splenic donor B cells (Ly5.1$^+$ B220$^+$ sIgM$^+$, 10.2% vs. 5.2%, $P = 0.006$) and a more modest (38%) relative increase in immature donor B cells (Ly5.1$^+$ B220$^+$ sIgM$^-$, 4.7% vs. 3.4%, P = ns) (Table 2). To determine the relative numbers of mature and immature splenic donor B cells in animals treated with SCH32615 or vehicle alone, the percentages of sIgM$^+$ and sIgM$^-$ cells among total Ly5.1$^+$ B220$^+$ splenocytes in the two groups of animals were compared (Fig. 3). Animals treated with the CD10/NEP inhibitor had relatively more mature (sIgM$^+$) donor-derived B cells and relatively less immature (sIgM$^-$) donor-derived B cells than vehicle-treated animals (sIgM$^+$, 70.3% vs. 58.3%, and sIgM$^-$, 29.7% vs. 41.7%, respectively, $P = 0.023$; Fig. 3). As a result, SCH32615-treated animals had a higher ratio of mature to immature B cells than animals treated with vehicle alone (2.4 vs. 1.4, ratio derived from Fig. 3). Taken together, these results suggest that CD10/NEP inhibition not only increases the reconstitution of splenic B cells but also promotes their maturation.

Effect of CD10/NEP Inhibition on Donor-Derived B-Cell Reconstitution in Lymph Nodes. In the congenic model, the effects of CD10/NEP inhibition were most apparent on donor-derived splenic B-cells (Tables 1 and 2; Fig. 3). Although there was a trend toward increased numbers of donor-derived B220$^+$ and sIgM$^+$ cells in the lymph nodes of SCH32615-treated animals, these differences did not reach statistical significance (Table 3). In these sublethally irradiated animals reconstituted with uncommitted hematopoietic progenitors, the spleen functions as one of the early sites of hematopoiesis in which donor cells expand and differentiate. In contrast, the lymph nodes in these animals are primarily composed of mature B cells that have migrated from areas of primary hematopoiesis. As expected, the lymph nodes in congenic animals contained predominantly mature B cells (Table 3), whereas spleens contained larger numbers of immature B-cell progenitors (Table 2). Since CD10/NEP is expressed by a restricted population of immature lymphoid progenitors (26), it is not surprising that inhibition of the enzyme had a more pronounced effect on the splenic lymphoid compartment.

Our results on the lymphoid differentiation of uncommitted hematopoietic progenitors in congenic animals treated with SCH32615 indicate that CD10/NEP inhibition promotes B-cell reconstitution and differentiation. Since inhibition of CD10/NEP results in increased local concentrations of relevant peptide substrate, these results further suggest that a peptide hydrolyzed by the enzyme stimulates the proliferation and differentiation of immature B-cell progenitors. Such a CD10/NEP peptide substrate is likely to have a unique role

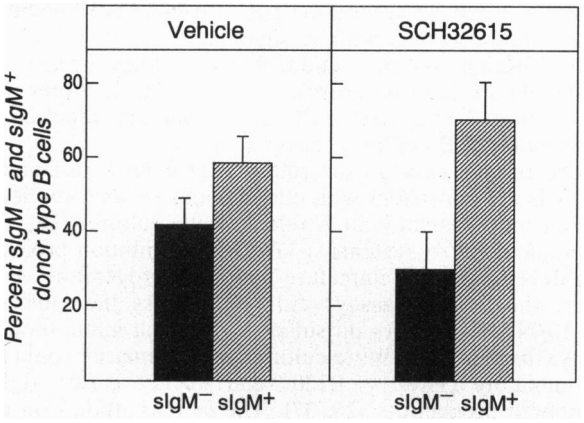

FIG. 3. Relative percentages of sIgM$^-$ and sIgM$^+$ Ly5.1$^+$ B220$^+$ spleen cells in Ly5.2 recipient animals treated with SCH32615 or vehicle alone. In SCH32615- and vehicle-treated animals from the experiment presented in Table 1, the percentages of sIgM$^-$ (■) and sIgM$^+$ (▨) cells among all Ly5.1$^+$ B220$^+$ cells are indicated. SCH32615-treated animals had relatively more mature (sIgM$^+$) donor-derived B cells and less immature (sIgM$^-$) donor-derived B cells than vehicle-treated animals (sIgM$^+$, 70.3% ± 9.9% vs. 58.3% ± 7.1%, and sIgM$^-$, 29.7% ± 9.9% vs. 41.7% ± 7.1% $P = 0.023$). Therefore, SCH32615-treated animals had a higher ratio of mature to immature B cells (70.2/29.7, ratio = 2.4) than animals treated with vehicle alone (58.3/41.6, ratio = 1.4).

Table 2. CD10/NEP inhibition increases the percentages of donor-derived mature B cells in spleen

Origin	Differentiation	% of cells		% change	*P* value
		Vehicle	SCH32615		
Ly5.1$^+$	B220$^+$ sIgM$^+$	5.2 ± 2.5	10.2 ± 3.6	+90	0.006
Ly5.1$^+$	B220$^+$ sIgM$^-$	3.4 ± 1.1	4.7 ± 2.6	+38	NS

Animals from the same representative experiment described in the legend to Table 1 were further characterized for donor-derived (Ly5.1$^+$) spleen cells expressing B220 and sIgM as described in the legend to Fig. 2 *B, C, E,* and *F*. In each congenic animal, the relative percentages of splenocytes expressing Ly5.1 and a combination of B220 and sIgM (B220$^+$ sIgM$^+$ or B220$^+$ sIgM$^-$) were determined. For the two groups of vehicle- and SCH32615-treated animals, the mean percentages (±SD) of spleen cells expressing each phenotype were calculated and the resulting values were compared using a one-sided Student *t* test. NS, not significant.

Table 3. Effect of CD10/NEP inhibition on donor-derived B cell reconstitution in lymph nodes

Origin	Lineage	% of cells		% change
		Vehicle	SCH32615	
Donor				
Ly5.1$^+$	All	10.9 ± 4.6	13.0 ± 3.3	+19
Ly5.1$^+$	B220$^+$	10.6 ± 4.6	12.8 ± 3.1	+21
Ly5.1$^+$	B220$^+$ sIgM$^+$	9.3 ± 4.0	11.3 ± 2.7	+22
Ly5.1$^+$	B220$^+$ sIgM$^-$	1.1 ± 0.6	1.1 ± 0.3	—

Animals from the same representative experiment described in the legends to Tables 1 and 2 were further characterized to determine the relative percentages of all lymph node cells expressing Ly5.1 and B220 or Ly5.1 and a combination of B220 and sIgM (as described in the legend to Fig. 2). For the two groups of vehicle- and SCH32615-treated animals, the mean percentages (±SD) of lymph node cells expressing each phenotype were calculated and the resulting values were compared using a one-sided Student *t* test.

in early B-cell differentiation because inhibition of the enzyme is associated with a significant increase in B-cell reconstitution and maturation. Recent studies, in fact, suggest that bone marrow stromal cells secrete factors other than interleukin 7 and stem cell factor that are required for maturation of B-cell progenitors (36).

The *in vivo* data on the role of CD10/NEP in congenic animals are consistent with our previous *in vitro* studies on the enzyme's function in Whitlock–Witte cultures (26). In *in vivo* and *in vitro* systems, CD10/NEP inhibition promoted the development of immature lymphoid progenitors. However, the *in vitro* assays did not address the effects of CD10/NEP inhibitors on subsequent B-cell maturation because the Whitlock–Witte cultures were primarily composed of immature (Thy-1^{low} B220^{+} and Thy-1^{-} B220^{+} sIgM^{-}) lymphoid progenitors (26, 37). The *in vivo* studies on congenic mice provide additional information on the effects of CD10/NEP inhibition on further B-cell differentiation. It is not yet possible to determine whether a putative CD10/NEP peptide substrate stimulates the proliferation of lymphoid progenitors resulting in the formation of increased numbers of mature B cells or whether the peptide substrate actually promotes B-cell differentiation.

Although CD10/NEP inhibition increased the relative percentages of donor-derived mature splenic B cells in congenic animals 21 days after the injection of uncommitted hematopoietic progenitors, the differences in donor B-cell percentages in SCH32615- and vehicle-treated animals decreased with subsequent follow-up (data not shown). In our earlier *in vitro* studies (26), the differences in lymphoid colony numbers in modified Whitlock–Witte cultures treated with CD10/NEP inhibitors or controls also decreased at later time points. These results suggest that the effects of CD10/NEP inhibition are most striking when a population of early hematopoietic progenitors undergoes rapid expansion. Alternatively, the effects of enzyme inhibition may become less apparent after prolonged treatment because of compensatory mechanisms. Since CD10/NEP hydrolyzes peptides that transmit signals after binding to specific cell-surface receptors, it is possible that long-term CD10/NEP inhibition results in decreased peptide secretion or down-regulation of the appropriate peptide receptor.

The fact that inhibition of CD10/NEP promotes the proliferation and maturation of lymphoid progenitors is also of interest because inhibition of the enzyme has similar effects in other organ systems. In recent studies, we demonstrated that CD10/NEP is primarily expressed by immature epithelial cells in the distal airways of developing fetal lung and that SCH32615-mediated CD10/NEP inhibition increases the proliferation and maturation of these immature epithelial cells (22, 32).

In conclusion, the current studies suggest that CD10/NEP and its peptide substrate play an important role in early B-cell differentiation. The studies provide a framework in which to further characterize the enzyme and its peptide substrate and to consider their roles in certain B-cell malignancies.

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