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Inhibitory effect of semaphorin-3A, a known axon guidance molecule, in the human thymocyte migration induced by CXCL12

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ABSTRACT

Intrathymic T cell differentiation takes place within the thymic lobules and depends on interactions between developing thymocytes and cells of the thymic microenvironment. Along with differentiation, thymocytes migrate in an oriented progression, which is tightly regulated by a number of interactions, including one mediated by the chemokine CXCL12. It has been shown recently that SEMA-3A, a soluble member of the semaphorin family, is also involved in this human thymocyte migration and can have a chemorepulsive and de-adhesive role. Herein, we study the role of SEMA-3A on the CXCL12-driven migration of human thymocytes. We have shown that SEMA-3A is able to inhibit the chemotaxis triggered by CXCL12. Such an inhibition was seen in respect to immature and mature CD4/CD8-defined thymocyte subsets and can be reverted specifically by neutralizing anti-SEMA-3A mAb. We have also shown that SEMA-3A consistently down-regulates CXCR4 membrane expression in all CD4/CD8-defined thymocyte subsets, and this down-regulation is accompanied by a decrease in the phosphorylation of FAK and ZAP-70 protein kinases. Taken together, these results demonstrate the involvement of SEMA-3A in the regulation of CXCL12-driven human thymocyte migration, where it acts as a physiological antagonist. *J. Leukoc. Biol.* 91: 7–13; 2012.

Introduction

The thymus is the lymphoid organ responsible for normal T cell development in vertebrates. This process is highly complex, including proliferation, cell death, and rearrangements of the TCR gene [1]. The thymic microenvironment is composed of ephitelial cells, DCs, and macrophages, which interact with thymocytes and provide a variety of signals for their maturation [2]. Initially, the bone marrow progenitor cells enter in the thymic lobules by corticomedullary junction. Then, the prethymocytes migrate toward the subcapsular region, where they receive inducing stimuli of proliferation and differentiation [3–5]. The sequential stages of the thymocyte differentiation progresses through the cortical and medullar regions of the thymic lobules. Ultimately, mature T cells are exported from the organ to the periphery of the immune system [6].

Moreover, during thymocytes development, these cells are marked by different expressions of cell surface molecules. In this context, the distinct profile of expression of CD4 and CD8 receptors on thymocytes is a useful indicator of the maturity in these cells. The thymocytes negative for the CD4 and CD8 expression are DN and are located mainly in the thymic subcapsular region. Analogously, the thymocytes that coexpress these molecules are DP and are located in the cortical region. Although thymocytes that only display the expression of one of these two types of molecules are SP thymocytes (SP4 or SP8, respectively), they are present in corticomedullar and medullar thymic regions [7].

Integrins [8], cytokines [9], sphingolipids [10], hormones [11], and chemokines [12–17] are some of the key factors that guide cell migration throughout the various anatomical niches within the thymic lobules [4]. These molecules act in combination, and the sum of their effects determines the resulting displacement of the thymic lymphoid cell. Particularly, several types of chemokines are expressed in the thymus. They regulate the migration of thymocytes by controlling integrin avidity and through the formation of chemotactic gradients [12–14]. Chemokines such as CCL25 and CCL22 mediate chemotaxis of

Abbreviations: APC=allophycocyanin, CNRS=National Center for Scientific Research, DN=double-negative, DP=double-positive, NP-1=neuropilin 1, SEMA-3A=semaphorin-3A, SP=single-positive, TEC=thymic epithelial cell

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immature thymocytes [15, 16], whereas CCL19 and CCL21 induce a recognized chemotactic effect on CD4 or CD8 SP thymocytes [15].

One of the chemotactic factors playing a major role in the intrathymic T cell migration in mice and humans is the chemokine CXCL12 (stromal cell-derived factor 1); it directs the migration of precursor cells from the corticomedullar region to the subcapsular zone [18] and the movement of the immature thymocytes through the cortical region of the thymic lobule. Moreover, the chemokine CXCL12 is involved in the emigration of mature T cells from the thymus to peripheral lymphoid organs [19, 20].

In the human thymus, CXCL12 is produced in high concentrations by TECs located in the subcapsular and medullar regions of the organ [18]. The receptor for CXCL12 is CXCR4 and is highly expressed in intrathymic progenitor cells and immature $CD4^+CD8^+$ thymocytes [18, 21]. The interaction between CXCL12 and CXCR4 triggers the activation of biochemical pathways in the intracellular environment [22], including tyrosine kinase-dependent events to cell migration [22–24]. One of these biochemical pathways described already reveals that the CXCL12/CXCR4 interaction triggers signal transduction pathways mediated by tyrosine phosphorylation of focal adhesion components, such as FAK [23, 25]. Moreover, other investigators showed that CXCL12-dependent T cell migration involves activation of the ZAP-70 tyrosine kinase [26, 27].

We have found recently that SEMA-3A mRNA and protein expressed by CD4/CD8-defined thymocytes are also involved in human thymocyte migration, where they exert a chemorepulsive and a de-adhesive effect [28].

SEMA-3A is a soluble member of the semaphorin family, known for its role in axonal guidance in the nervous system. However, the spectrum of activities of semaphorins and their receptors has been found increasingly widespread, as these molecules participate in distinct biological processes, such as mobility of vascular endothelial cells and lung-branching morphogenesis [29]. There are two main families of transmembrane proteins forming the receptor for semaphorins, which are the NPs and the plexins. NPs associate with plexins in the semaphorin receptor formation. Although the binding site of semaphorins may be the only NP [30], plexins are mainly responsible for the internal transmission of the signal generated in the cell membrane, as these proteins have a long cytoplas-

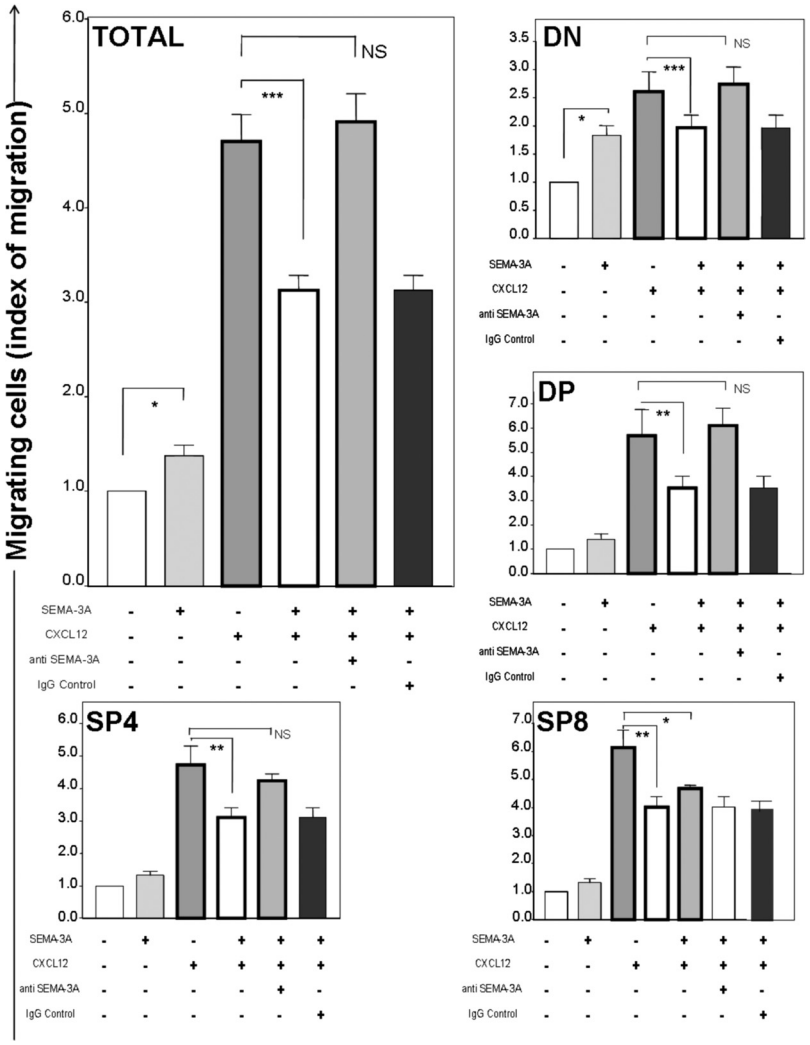

Figure 1. SEMA-3A down-regulation of CXCL12-driven human thymocyte migration. Ex vivo experiments performed in Transwell cell migration chambers show that SEMA-3A per se is chemorepulsive when administered into the upper chamber of the Transwell system. Moreover, it partially impairs the chemoattraction of human thymocytes induced by CXCL12 in the lower well. The specificity of the effect of SEMA-3A was proved by its reversion by a specific anti-SEMA-3A antibody (but not by an unrelated reagent). This effect can be seen in total thymocytes, as well as in each CD4/CD8-defined subset. These data are from 23 separate experiments. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

mic domain, in contrast to the NPs that have a short intracellular extension [30].

In the present work, we have investigated the role of SEMA-3A on the CXCL12-driven migration of human thymocytes. We have shown that SEMA-3A is able to inhibit the chemotaxis triggered by CXCL12, thus acting as a physiological antagonist for this chemokine-driven migration.

MATERIALS AND METHODS

Human thymocyte preparations

Human thymic tissue was obtained from children, from 5 days to 3 years of age, undergoing cardiac surgery. Experimental procedures with human thymic fragments have been approved in Brazil by the Oswaldo Cruz Foundation and in Paris by the Necker Hospital Ethical Committee for human research and were conducted according to the guidelines of the European Union and the Helsinki Declaration. Thymocyte cell suspensions were prepared by gently pressing thymus lobes as described previously [31].

Antibodies and recombinant proteins

Mouse mAb used in flow cytometry were the anti-NP-1 reagent blood DC antigen-4-PE or -APC (Miltenyï Biotec, Bergisch Gladbach, Germany); anti-CXCR4-PE or -FITC (PharMingen/Becton Dickinson, San Diego, CA, USA); and anti-CD8-FITC, -PE, or -APC and anti-CD4-FITC or -PE-cyanine 5, along with the appropriate Ig isotype controls (Immunotech, Marseille, Cotê D'Azur, France). The anti-SEMA-3A mouse mAb was furnished by Anat Shirvan and Ari Barzilai (Felsenstein Medical Research Center, Petah Tikva, Israel). For the immunofluorescence assays, anti-SEMA-3A antibody from Santa Cruz Biotechnology (Santa Cruz, CA, USA) was used, whereas the anti-CXCL12 was purchased from R&D Systems (Minneapolis, MN, USA). The presence of the anti-CXCL12 antibody was revealed by a biotinylated donkey anti-mouse Ig (Amersham Bioscience, GE Healthcare Bio-Sciences, Piscataway, NJ, USA), followed by streptavidin/Alexa 488 (Molecular Probes, Eugene, OR, USA), whereas the anti-SEMA-3A reagent was detected with a goat anti-rabbit Ig serum coupled to PE. Antiphospho-ZAP-70 rabbit polyclonal antibodies were from Cell Signaling Technology (Danvers, MA, USA). The antiactin goat polyclonal antibody was from Santa Cruz Biotechnology, and the antiphospho-FAK rabbit polyclonal antibody was from Sigma-Aldrich (St. Louis, MO, USA). Human rFc-SEMA-3A and CXCL12 were purchased from R&D Systems.

Chemotaxis assay

Thymocyte migratory activity was assessed ex vivo in 5 μm pore-size transwell plates (Corning Costar, Lowell, MA, USA), as reported previously [31]. Thymocytes (2.5×10^6) with SEMA-3A (50 ng/mL) were plated in the upper chamber in 100 μl RPMI 1640/0.5% BSA, and 600 μl RPMI 1640/0.5% BSA was added to the lower chamber, with or without CXCL12 (100 ng/mL). In the experiments of preincubation, the cells were incubated with SEMA-3A for 30 min, washed, and submitted to migration. After 3 h, the cells that migrated into the lower chambers were counted and immunostained for the detection of NP-1, CXCR4, CD4, and CD8.

Immunofluorescence and confocal microscopy

Frozen sections of the thymus were double-labeled according to routine procedures. Specimens were subjected to anti-SEMA-3A and anti-CXCL12 antibodies, followed by PE-labeled goat anti-mouse IgG and by Alexa-488-labeled goat anti-rabbit IgG, respectively. Analyses were conducted by confocal microscopy.

Immunoblotting Human thymocytes (10^7 cells) were incubated in RPMI-1640 medium, with or without SEMA-3A (50 ng/mL) and CXCL12 (100 ng/mL). Cells were harvested, washed with ice-cold PBS, and lysed in 250 μL boiling sample lysis buffer (0.15 M NaCl, 0.05 M Tris-HCl, pH 7.2, 1% Triton X-100, 1% sodium deoxycholate, 2% SDS) containing protease and phosphatase inhibitors (10 μg/mL aprotinin, 10 μg/mL leupeptin, 10 μg/mL benzamidin, 10 μg/mL pepstatin). Crude lysates were obtained by centrifugation to pellet nuclei. Protein content was measured using the BioRad protein assay (Pierce, Northumberland, UK). Equal amounts of

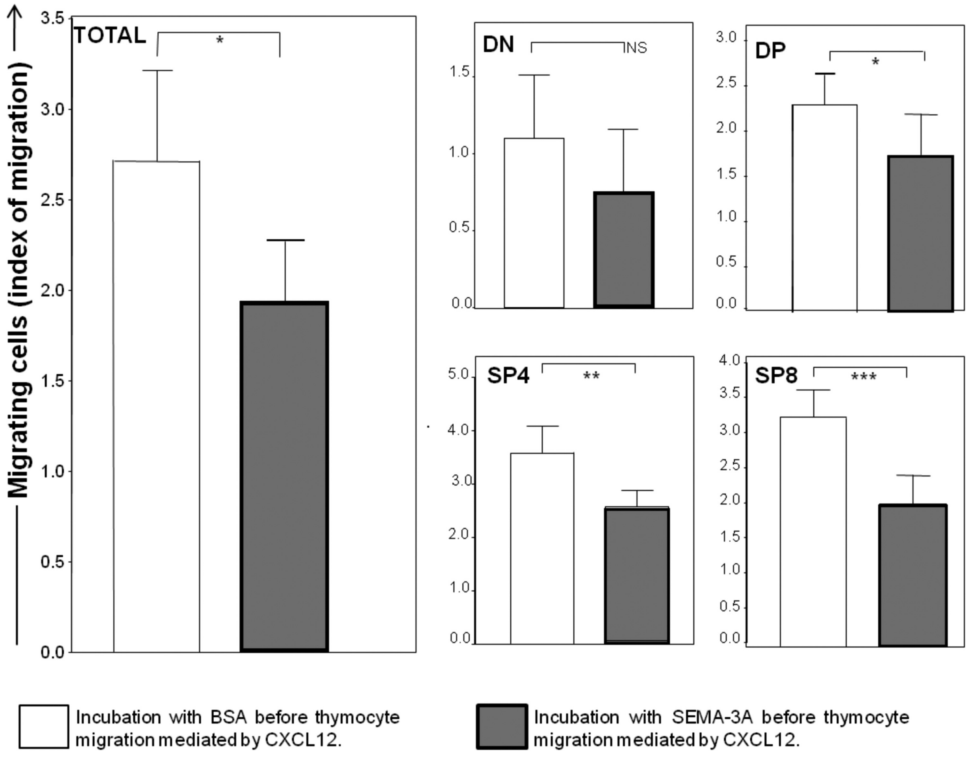

Figure 2. Impairment of CXCL12-driven thymocyte migration by preincubation with SEMA-3A. The preincubation of thymocytes with SEMA-3A, followed by PBS washing prior to migration toward CXCL12, is sufficient to significantly impair CXCL12-driven migration. This effect can be seen in bulk thymocytes and in each CD4/CD8-defined subset. These data are from 25 separate experiments. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

protein were loaded on a 10% SDS-PAGE and transferred to PVDF membrane (Amersham Bioscience). Primary antibodies were revealed with an appropriate peroxidase-coupled secondary antibody and detected by an ECL kit (Pierce). Blots were also probed with an antiactin polyclonal antibody (Santa Cruz Biotechnology) to confirm equal loading of protein.

Statistical analysis

Data were statistically evaluated using the Wilcoxon test for nonparametric paired samples. Differences were considered statistically significant when $P \leq 0.05$.

RESULTS

SEMA-3A down-regulation of CXCL12-driven human thymocyte migration

Application of SEMA-3A into the upper chamber of a transwell device was found to inhibit CXCL12-driven thymocyte chemotactic migration significantly (**Fig. 1**). This inhibition affected immature and mature CD4/CD8-defined thymocyte subsets and could be reverted specifically by anti-SEMA-3A-neutralizing mAb. Moreover, preincubation of thymocytes with SEMA-3A was sufficient to inhibit CXCL12-mediated migration significantly (**Fig. 2**). However, in similar assays performed with others chemokines, the SEMA-3A did not inhibit the correlated chemotaxis (see **Fig. 3**), thus indicating specificity of the inhibitory effect of the SEMA-3A on the chemokine-driven human thymocyte migration. Of note, even the chemorepulsive role of CXCL12 on thymocytes, seen when this chemokine is applied in high concentrations [32], was also abrogated significantly by SEMA-3A (data not shown).

Taken together, these results show for the first time that SEMA-3A is involved in the chemotaxis of CXCL12-triggered thymocyte migration responses.

SEMA-3A decrease of CXCR4 expression on thymocytes

Considering the effects described above, it was conceivable that SEMA-3A might alter the CXCL12-driven migration by acting through the corresponding receptor, CXCR4. It was found that SEMA-3A applied in the upper transwell chambers did indeed down-regulate CXCR4 membrane expression consistently and significantly for almost all CD4/CD8-defined thymocyte subsets. CXCL12 also caused down-regulation of the CXCR4 receptor [33]. Interestingly, when SEMA-3A was applied in conjunction with CXCL12, the membrane density of CXCR4 decreased even further (**Fig. 4**).

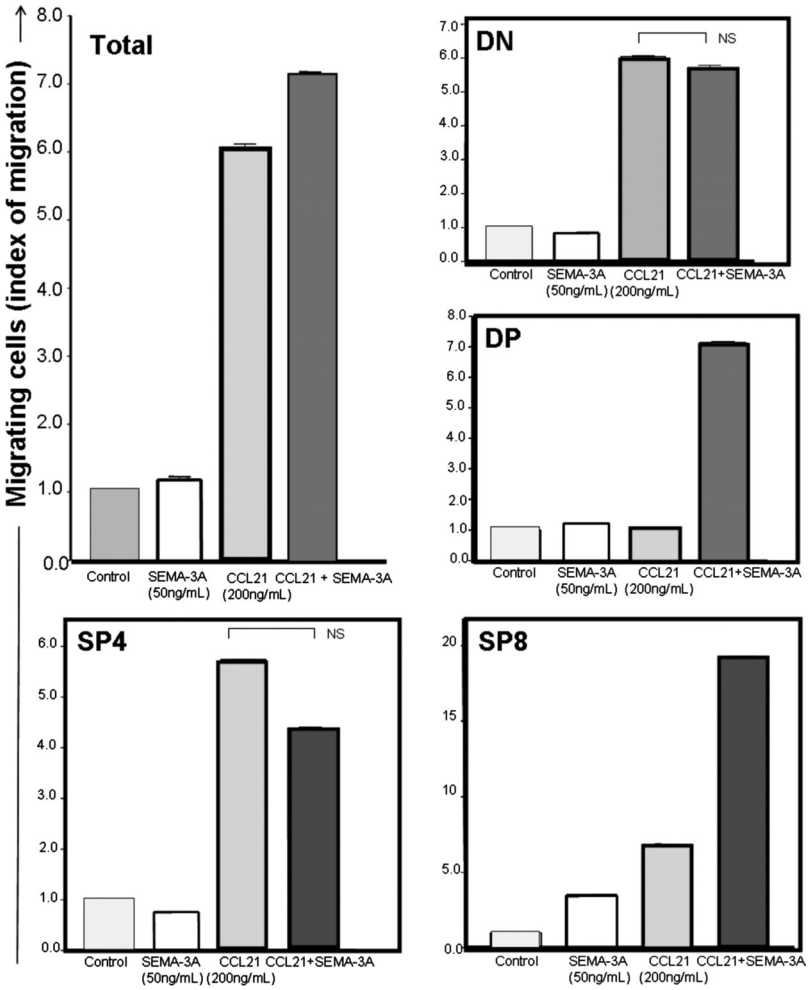

Figure 3. SEMA-3A does not inhibit the migration of human thymocytes mediated by CCL21. The copresence of SEMA-3A induced an increase in total, DP, and SP8 thymocyte migration and did not affect the migration of DN and SP4 thymocytes directly by this chemokine. These results suggest that the migration of total, DP, and SP8 subsets was caused by a synergistic effect of the SEMA-3A and CCL21.

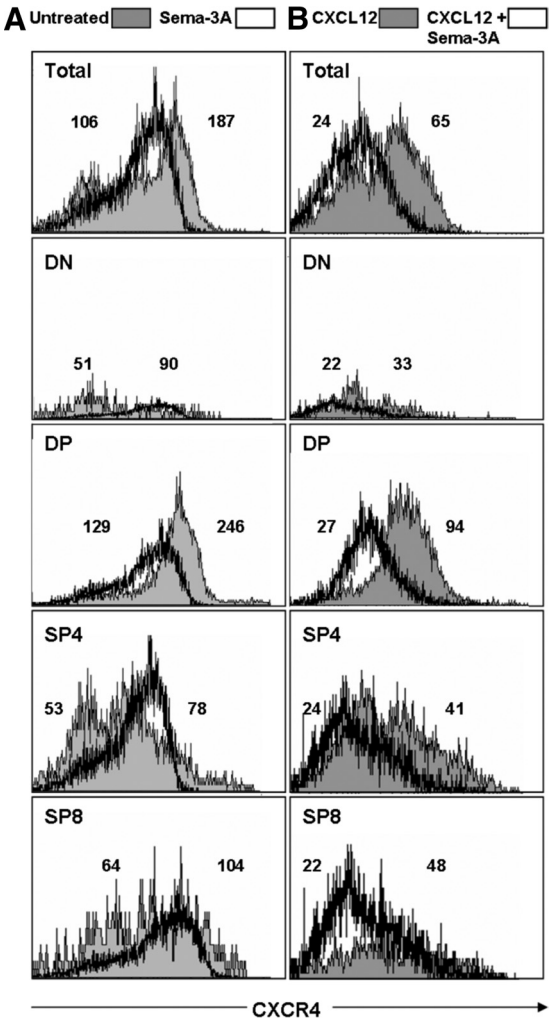

Figure 4. SEMA-3A-induced decrease in CXCR4 expression in human thymocyte subsets. This effect can be seen in total thymocytes as well as in the CD4/CD8-defined subsets. In the representative experiments illustrated here, each number corresponds to the mean fluorescent intensity generated by anti-CXCR4 labeling. The numbers on the right side of the panels correspond to the absence of SEMA-3A, whereas those on the left were generated when thymocytes were incubated with SEMA-3A. (A) Incubation of thymocyte suspensions with SEMA-3A (50 ng/mL) per se for 1 h, followed by extensive washing, was able to down-regulate the CXCL12 receptor, CXCR4. Total: $P < 0.05$; DN: NS; DP: $P < 0.05$; SP4: $P < 0.005$; SP8: $P \leq 0.001$. (B) The simultaneous coincubation of CXCL12 and SEMA-3A resulted in a more drastic reduction in the CXCR4 density of expression than that induced by chemokine alone. Total: $P \leq 0.05$; DN: 0.05; DP: $P \leq 0.005$; SP4: $P \leq 0.05$; SP8: $P \leq 0.05$. Data are representative of 25 independent experiments.

SEMA-3A induces the modification of signal transduction pathways triggered by a CXCL12/CXCR4 interaction

A CXCL12/CXCR4 interaction is known to trigger downstream pathways that include activation of tyrosine kinases, such as the FAK [25] and the tyrosine kinase ZAP-70 [26, 27]. The activation of FAK and ZAP-70 in turn depends on its prior phosphorylation.

FAK phosphorylates focal adhesion proteins, which are cytoskeletal structures that interact with components of the ECM; their phosphorylation is associated with cellular mobilization induced by chemotactic factors [34]. As for the ZAP-70, Nardine and collaborators [26] showed that ZAP-70-dependent tyrosine phosphorylation is involved in CXCR4 chemokine receptor signaling in human T cells. We thus analyzed the activity of these two kinases by analyzing their levels of phosphorylation in thymocytes treated with CXCL12 and SEMA-3A, alone and in combination. Immunoblotting experiments revealed that SEMA-3A decreases the phosphorylation of FAK and ZAP-70 in thymocytes treated and untreated with CXCL12 (**Fig. 5A**). These results were analyzed further by cytofluorometry (Fig. 5B and C, respectively). Again, this effect was seen in the CD4/CD8-defined thymocyte subsets evaluated.

Taken together, these results indicate that SEMA-3A seems to decrease the phosphorylation status of FAK, which could explain, at least in part, the intracellular mechanisms involved in the inhibitory action of SEMA-3A on CXCL12-mediated human thymocyte migration. In addition to FAK, we observed that ZAP-70 phosphorylation was reduced by SEMA-3A in all CD4/CD8-defined subsets of human thymocytes, thus showing that one mechanism by which SEMA-3A exerts its inhibitory effect is through changes in the phosphorylation status of in-

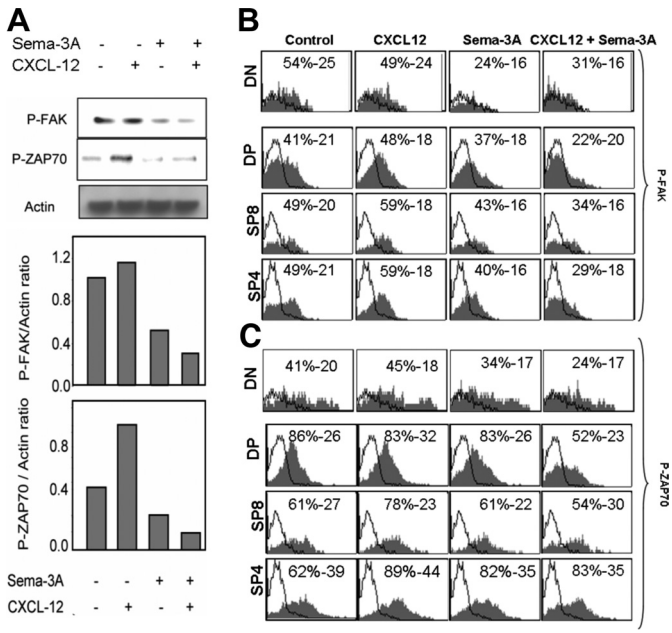

Figure 5. SEMA-3A down-regulation of CXCR4-induced downstream signaling pathways in human thymocytes. (A) Immunoblots for detection of phosphorylation of FAK (P-FAK) and ZAP-70 (P-ZAP-70) protein kinases. Actin labeling was used as a control for the expression of a housekeeping gene. The corresponding histograms represent the densitometry of each blot (discounting the corresponding actin labeling). SEMA-3A clearly impairs CXCL12-triggered phosphorylation of the two protein kinases. (B and C) The cytofluorometric profiles of CD4/CD8-defined human thymocyte subsets for the detection of the same phosphorylated proteins, FAK and ZAP-70, respectively. Data are representative of at least three independent experiments.

tracellular signaling molecules triggered by CXCR4 stimulation.

Colocalization of SEMA-3A and CXCL12 in human thymic tissue

In addition to the functional assays showing the ex vivo effects of SEMA-3A on CXCL12-driven human thymocyte migration, it was necessary to demonstrate that these molecules coexist inside the organ, in the same place at the same time. Double-labeling immunofluorescence experiments were thus conducted on frozen sections of the human thymus, using anti-SEMA-3A- and anti-CXCL12-specific antibodies, with specimens being evaluated by confocal microscopy and further computational analysis. The molecules were in fact colocalized in cortical and medullar regions of the thymic lobules (**Fig. 6A**). Such colocalization coincides with the profile of the TEC network, although the concentration of SEMA-3A in the inner cortex is much greater as a result of production by TEC and thymocytes, as reported previously [31]. The relative intensity of labeling of each marker is shown graphically as a function of the number of pixels recorded for each point (Fig. 6B). Thymic colocalization of SEMA-3A and CXCL12 was evaluated with the use of Mander's coefficient [35]. According to Mander's coefficient, values vary from zero (no colocalization) to one (total colocalization of pixels). The values obtained for the colocalization of the two proteins analyzed were 0.8 in the total area of the cortical region and 0.9 in the medullary region. Despite the extensive colocalization of the ligands, the combined flow cytometry analysis of CXCL12 and SEMA-3A receptors (Fig. 6C) reveals only a small proportion of cells in each.

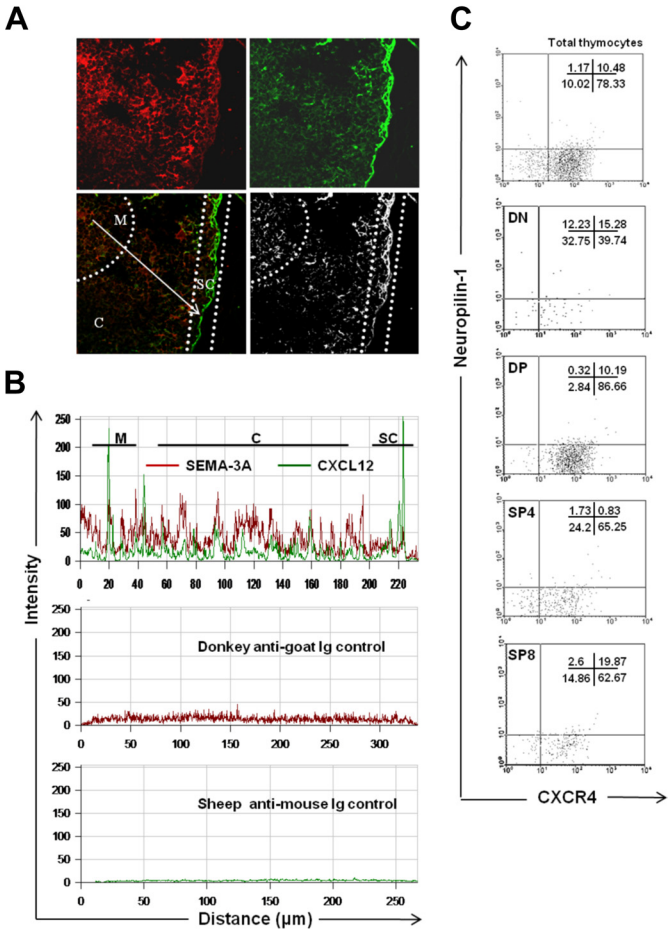

Figure 6. Colocalization of SEMA-3A and CXCL12 inside human thymic tissue. This figure depicts a section of human thymus double-labeled for the immunohistochemical detection of SEMA-3A (red) and CXCL12 (green). (A) The microscopic field shows that both molecules are largely colocalized in the subcapsular (SC), inner-cortical (C), and medullar (M) regions of the thymic lobule (as evaluated by Mander's coefficient for total area and specific regions, with colocalization of SEMA-3A and CXCL12 of 80% and 90%, respectively). This colocalization is seen further in the lower right picture of the panel, in which a computer-generated signal (seen in white) traces all sites of colocalization, which was evaluated further by computational analysis, in which the density for each labeling marker was scanned, and the numbers of pixels transformed into intensity in histograms. (B) Each specific histogram (red for SEMA-3A and green for CXCL12) derives from the scanning of the microscopic field traced with the arrows seen in A, with distances expressed in micrometers. Again, colocalization is apparent in cortical and medullar regions. Negative controls for specific antibodies did not generate any significant signal, as shown in the middle and bottom graphs. (C) CXCR4 and NP-1 expression in human thymocytes.

DISCUSSION

Taken together, the information derived from these ex vivo experiments shows that the SEMA-3A modifies the migration of all subpopulations of thymocytes defined by expression of CD4 and CD8 molecules. This was supported further by results shown in in situ coexpression of CXCL12 and SEMA-3A in the human thymic lobules—the cortex and the medulla. It is thus plausible to suggest that within the normal human thymus, these molecules could be acting simultaneously upon thymocytes at their various developmental stages.

Conceptually, these data indicate that SEMA-3A plays a major regulatory role in the oriented movement of developing T cells within the human thymus.

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DISLCOSURE

There are no conflicting interests.

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