Control of human thymocyte migration by Neuropilin-1/Semaphorin-3A-mediated interactions

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It is largely established that molecules first discovered in the nervous system are also found in the immune system. Neuropilin-1 (NP-1) was initially identified to mediate semaphorin-induced chemorepulsion during brain development and is also involved in peripheral T cell/dendritic cell interactions. Herein, we studied NP-1 during T cell development in the human thymus. NP-1 is expressed in both cortex and medulla of thymic lobules, being found in distinct CD4/CD8-defined thymocyte subsets. NP-1 is also found in thymic epithelial cells (TEC) *in situ* and *in vitro*, and is recruited at the site of TEC–thymocyte contact. Moreover, NP-1 was rapidly up-regulated during thymocyte stimulation by T cell receptor (TCR) and IL-7 or after adhesion to TEC. Semaphorin-3A (Sema-3A), a natural ligand of NP-1, is also present in human thymus, both in TEC and thymocytes, being up-regulated in thymocytes after TCR engagement. Functionally, Sema-3A decreases the adhesion capacity of NP-1$^+$ thymocytes and induces their migration by a repulsive effect. In conclusion, we show here that NP-1/Sema-3A-mediated interactions participate in the control of human thymocyte development.

extracellular matrix | integrins | thymocyte adhesion and migration

Migration of thymocytes is a crucial event in intrathymic T cell differentiation. At least two large families of molecules have been implicated as playing a role in thymocyte migration: extracellular matrix (ECM) proteins and chemokines (1–3). Moreover, recent data point to a combined effect of ECM and chemokines on thymocyte migration (3, 4). Nevertheless, one can expect that such migration is under a broader control that comprises yet unknown molecular interactions.

It is established that molecules typically discovered in the nervous system are also found in the immune system and vice versa (5). For example, the chemokine CXCL12 is able to drive neuron migration (6), whereas stem cell factor controls neuron migration through CD117/c-*kit* activation (7). Accordingly, we might expect that interactions involved in cell migration within the nervous tissues also exist in the immune system, more particularly in the thymus. The candidate approached herein is neuropilin-1 (NP-1), a 130-kDa transmembrane protein receptor, initially identified to mediate the chemorepulsive activity of semaphorins during embryonic brain development (8, 9). Semaphorins correspond to a large family of transmembrane and secreted glycoproteins that function in repulsive growth cone and axon guidance. NP-1 interacts directly with one member of the semaphorin family, semaphorin-3A (Sema-3A) (9), and induces cytoskeleton changes ultimately driving repulsion of axons (10). Additionally, it is expressed in endothelial cells, playing a role in angiogenesis (11). We have shown that NP-1 is expressed on dendritic cells (DCs) and peripheral T cells (12). At this level, NP-1 seems to be involved in the immunological synapse formation and colocalized with the T cell receptor (TCR) on T cells, during DC–T cell contact (12). In angiogenesis, Sema-3A was shown to inhibit adhesion of endothelial cells, indicating a role in the migratory activity of this cell type (13). All of these data prompted us to study the putative role of NP-1/Sema-3A interaction on human thymocyte adhesion and migration.

We show here that NP-1 and Sema-3A are constitutively expressed in the human thymus in both thymic epithelial cells (TEC) and CD4/CD8-defined thymocytes. TEC–thymocyte adhesion enhances NP-1 expression on thymocytes. This effect may be partially attributed to IL-7 secreted by TEC and to TCR engagement, because both stimuli enhance NP-1 surface expression on thymocytes. Moreover, NP-1-mediated thymocyte adhesion is inhibited by Sema-3A, and this activity is mainly because of the decrease in the integrin-mediated adhesion capacity of thymocytes on ECM substrata. Lastly, Sema-3A induces a chemorepulsive activity on thymocytes and on thymic DC.

Results

Human Thymic Cells Constitutively Express NP-1. We first defined the constitutive expression of NP-1 in the human thymus. *In situ* immunohistochemistry revealed that epithelial (cytokeratin-containing) cells express NP-1 (Fig. 1*A*). High membrane levels of NP-1 were also found in cultures of fetal and postnatal human TEC lines (Fig. 1*B*), freshly isolated thymic nurse cells (TNC), and primary cultures of TNC-derived epithelium (Fig. 1*C*). NP-1 gene expression by TEC was confirmed by RT-PCR (Fig. 1*D*). In contrast, only a minority of thymocytes express NP-1 constitutively, although NP-1$^+$ cells were detected in all CD4/CD8-defined subsets (Fig. 1*E*). Percentage of NP-1$^+$ cells within each subset varied among the thymuses evaluated ($n = 19$), without any correlation with age or sex of the infant (Table 1). NP-1$^+$ thymocytes ranged from 1.32 to 12.68% with an average of 5.11%. Finally, CD13$^+$/CD11c$^+$ afferent myeloid thymic DCs (14, 15) also express NP-1, bearing a higher membrane density than thymocytes (data not shown).

IL-7 and TCR Engagement Up-Regulate NP-1. TEC constitutively secrete IL-7, which is crucial for progression of very immature thymocytes (16). When we preincubated thymocytes with IL-7, we observed an up-regulation of NP-1 expression in thymocytes, as ascertained by cytofluorometry and RT-PCR (Fig. 2), as early as 6 h of IL-7 exposure. Such an increase was seen not only in immature thymocytes but also in mature CD4$^+$ and CD8$^+$ single-positive subsets (Fig. 2*A*).

In addition, NP-1 expression increased on thymocytes during

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Abbreviations: ECM, extracellular matrix; NP-1, Neuropilin-1; Sema-3A, semaphorin-3A; DC, dendritic cell; TCR, T cell receptor; TEC, thymic epithelial cell; TNC, thymic nurse cell; VLA, very late activation antigen; mAb, monoclonal antibody; PE, phycoerythrin.

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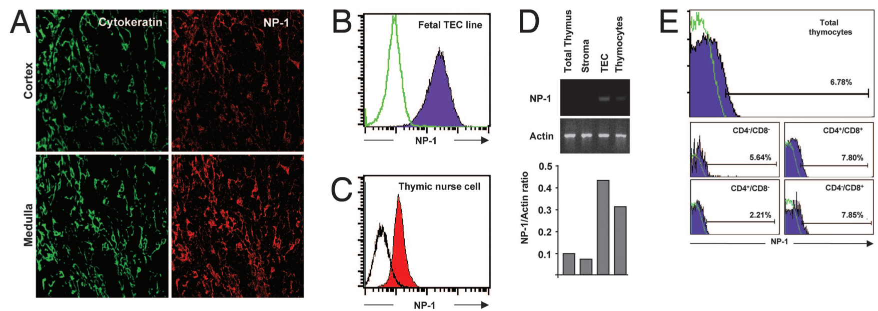

Fig. 1. NP-1 expression in the human thymus and thymic cell types. (*A*) *In situ* expression of NP-1 on thymus in both cortex and medulla. Note that most of NP-1$^+$ cells shown in this microscopic field correspond to TEC selectively revealed herein with an anti-cytokeratin antibody. (Original magnification, ×250.) (*B* and *C*) Flow cytometry profiles showing that both fetal TEC lines and TNC express NP-1. (*D*) Semiquantitative PCR showing NP-1 expression on thymus, total stroma, TEC, and thymocytes. Histograms represent the NP-1/actin ratio and shows the pattern expression of NP-1 mRNA. (*E*) Thymocyte subsets, defined by CD4, CD8 staining into double-negative (CD4$^-$/CD8$^-$), double-positive (CD4$^+$/CD8$^+$), and CD4 and CD8 single-positive cells (CD4$^+$/CD8$^-$, CD4$^-$/CD8$^+$), show similar pattern of NP-1 expression. Percentages illustrate the relative number of NP-1$^+$ thymocytes in each CD4/CD8-defined subset.

TEC–thymocyte contact, suggesting that cell–cell interaction participates in NP-1 regulation (Fig. 3*A*). Moreover, IL-7 positively contributed to the increase in NP-1 expression (Fig. 3*A*). To better define the involvement of this contact on NP-1 up-regulation, we performed TCR stimulation to mimic cell–cell interaction, by subjecting thymocytes to the activator anti-TCR antibody (17). TCR engagement enhanced NP-1 expression, as rapidly as 3 h, as seen at the mRNA and protein levels (Fig. 3 *B* and *C*). These results suggest an involvement of NP-1 in thymocyte–TEC adhesion. Accordingly, thymocytes that adhered to TEC cultures were higher NP-1 expressors (Fig. 3*A*), as seen by confocal microscopy (Fig. 3*D*) and flow cytometry evaluation of adherent versus nonadherent thymocytes (Fig. 3*A*). Interestingly, confocal analysis of thymocyte–TEC cocultures showed that NP-1 is concentrated at the thymocyte pole that is in close contact with TEC (Fig. 3*E*). Taken together, these findings indicate that NP-1 is involved in thymocyte–TEC interactions.

Human Thymic Cells Express Sema-3A. To better understand the effect of NP-1 on thymocyte–TEC adhesion, it was necessary to determine whether its natural ligand, Sema-3A, is also expressed in the thymus. We found Sema-3A mRNA and protein expressed by human TEC *in situ*, both in cortex and medulla, as well as TEC cultures (Fig. 4 *A–C*). Interestingly, we found 80–95% of total thymocytes bearing cytoplasmic Sema-3A, comprising immature and mature subsets (Fig. 4*D*).

We then evaluated whether Sema-3A production by thymocytes could be also modulated by IL-7 or TCR engagement, as was the case for NP-1. Semiquantitative analysis of Sema-3A gene expression by thymocytes revealed only a minor up-regulation of mRNA on IL-7 stimulation (data not shown). By contrast, antibody-induced TCR engagement rapidly increased Sema-3A mRNA, a progressive effect that lasted for at least 24 h (Fig. 4*E*).

Sema-3A Regulates Thymocyte–TEC Contact. To further determine whether NP-1 participates in thymocyte–TEC adhesion, we preincubated thymocytes with Sema-3A. This procedure resulted in 50% inhibition of thymocyte adhesion, in a dose-dependent manner (Fig. 5*A*). Nonetheless, the CD4/CD8-defined phenotype of the adhered thymocytes was similar in the presence or absence of Sema-3A. This finding suggests that, at all stages of human thymocyte differentiation, adhesion to the thymic epithelium is under the influence of NP-1/Sema-3A-mediated interactions.

Given the results showing that NP-1 is involved in TEC–thymocyte adhesion, and that such an adhesion is partially abrogated by Sema-3A, we determined the NP-1 phenotype of adherent and nonadherent CD4/CD8-defined thymocyte subsets. Sema-3A decreased adhesion of NP-1$^+$ thymocytes and enriched the nonadherent fraction on NP-1$^+$ thymocytes in a dose-dependent

Table 1. Relative numbers of NP-1 cells in CD4/CD8-defined thymocyte subsets

Thymus	Age	Total	CD4$^-$/CD8$^-$	CD4$^+$/CD8$^+$	CD4$^+$/CD8$^-$	CD8$^+$/CD4$^-$
1	1 day	8.01	4.63	9.07	2.68	9.21
2	6 days	3.76	15.26	3.95	19.79	24.01
3	9 days	1.61	13.83	8.40	6.92	9.27
4	10 days	2.31	11.41	1.46	11.42	8.42
5	17 days	12.68	13.25	13.26	12.24	7.51
6	3 months	3.55	3.14	3.60	3.67	2.56
7	3 months	1.32	23.21	1.56	17.05	7.93
8	4 months	6.80	1.70	7.71	3.80	2.24
9	4 months	2.61	16.19	3.17	5.25	14.52
10	4 months	2.95	4.08	2.63	4.32	4.57
11	8 months	8.80	4.87	9.86	5.55	11.09
12	2 years	12.22	16.81	14.40	7.46	9.09
13	3 years	4.17	22.35	3.91	12.57	5.48
14	3 years	6.86	2.80	7.27	1.53	14.88
15	3 years	2.63	9.92	1.63	21.30	5.85
16	5 years	4.09	0.87	4.47	0.62	4.27
17	6 years	1.57	14.72	1.91	14.82	19.26
18	8 years	6.69	14.90	7.47	4.01	6.26
19	9 years	4.38	6.14	4.68	3.81	1.85
	Mean	5.11	10.53	5.81	8.36	8.86
	SEM	1.17	2.42	1.33	1.92	2.04

This table represents the percentage of total NP-1$^+$ and NP-1$^+$ CD4/CD8-defined thymocyte subsets obtained in fresh thymus samples from 19 young children (ages are between 1 day and 9 years). Mean and SEM of NP-1 expression on total and CD4/CD8-defined thymocyte subsets are indicated at the bottom of the table.

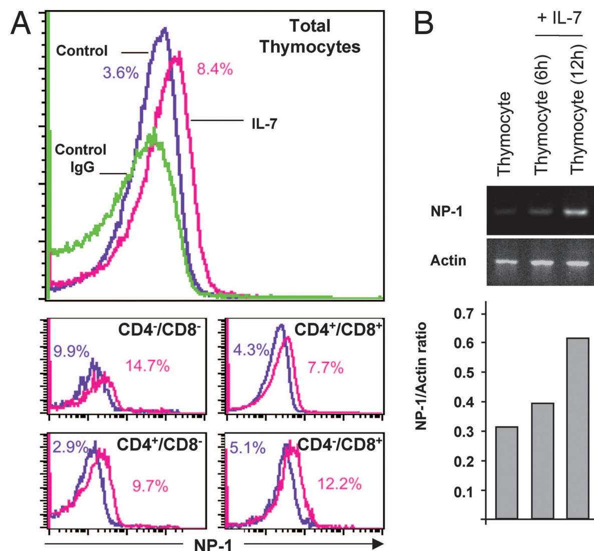

Fig. 2. IL-7 up-regulates NP-1 expression on thymocytes. (*A*) Flow cytometry analysis of NP-1 expression on IL-7-treated (red histograms) or untreated thymocyte subsets (blue histograms). The green curve corresponds to the labeling generated by an irrelevant fluorochrome-matched IgG. Up-regulation of NP-1 by IL-7 is seen in total thymocyte profile, as well as in each CD4/CD8-defined thymocyte subset. (*B*) Semiquantitative PCR showing NP-1 expression by IL-7-treated (during 6 and 12 h) or untreated (T0) total thymocytes. Histograms represent the NP-1/actin ratio and show the pattern of NP-1 mRNA expression. Data shown in each image are representative of three independent experiments.

manner (Fig. 5*B*). In addition, adhesion of enriched NP-1$^+$ thymocytes was partially inhibited by Sema-3A when compared with total or NP-1$^-$ thymocytes (Fig. 5*C*). These results represent evidence of a negative effect of Sema-3A on the NP-1$^+$ thymocyte adhesion on TEC, and the involvement of NP-1/Sema-3A complex on heterocellular interactions in the human thymus.

Chemorepulsive Effect of Sema-3A on Thymocytes. Because adhesion and de-adhesion are crucial for cell migration, and considering that Sema-3A exhibited a de-adhesive role on thymocyte–TEC adhesion, we searched whether this molecule could exert a chemorepulsive effect on thymocyte migration. Preincubation of thymocytes with Sema-3A in the upper chamber of Transwell plates did induce a dose-dependent enhancement of migration compared with BSA alone (Fig. 6*A*). By contrast, when Sema-3A was applied in the bottom well of the Transwell chambers (to mimic a typical chemoattraction), no effect was seen on thymocyte migration (Fig. 6*B*). A putative cooperation between chemoattraction of ECM (laminin or fibronectin) and chemorepulsion of Sema-3A was tested in similar conditions. As expected, laminin or fibronectin induced thymocyte attraction. However, when one of these stimuli was applied in combination with Sema-3A, resulting migration values were inferior to the sum of each stimulus alone (Fig. 6*C*). Lastly, we performed experiments on slides bearing a horizontal migration chamber. Sema-3A addition to thymocytes on a given side of the slide largely increased the migration toward the opposite side (Fig. 6*D*).

Sema-3A Inhibits Thymocyte Migration Through ECM Substrata. In accordance to the chemorepulsive and de-adhesion effects of Sema-3A on thymocytes, we investigated whether Sema-3A could modulate ECM-driven cell migration. As previously defined in thymus, very late antigen (VLA)-4, -5, and -6 are able to bind fibronectin and laminin, respectively, and participate in thymocyte–TEC adhesion (3). We incubated thymocytes or TEC with Sema-3A and checked for VLA expression. As ascertained by

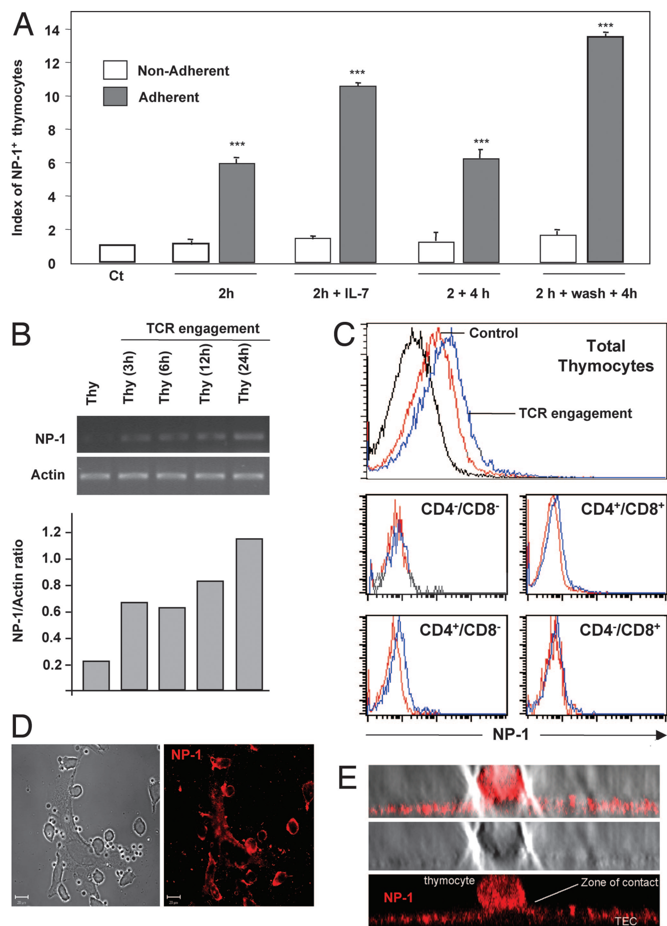

Fig. 3. NP-1 is involved in TEC–thymocyte adhesion. (*A*) TEC–thymocyte adhesion increases NP-1 expression on adherent thymocytes (2 h). IL-7 addition in this coculture context further enhances NP-1 expression on adherent thymocytes (2 h). Moreover, adherent thymocytes, cocultured for an additional 4 h, continue to increase NP-1 expression. This effect is even more prominent when the second coculture period is preceded by wash. (*B*) Semiquantitative PCR showing increase in NP-1 gene expression in thymocytes after TCR engagement. Histograms represent the NP-1/actin ratio. (*C*) Flow cytometry analysis of NP-1 expression on TCR stimulated (blue histograms) and untreated thymocyte subsets (red histograms) compared with irrelevant antibody (black histograms). (*D*) TEC–thymocyte coculture was stained by the anti-NP-1 antibody and analyzed by confocal microscopy. (Original magnification, ×250.) (*E*) NP-1 polarization at the TEC–thymocyte contact was observed by using *Z*-stack analysis by confocal microscopy. (Original magnification, ×1,000.) Data shown in each image are representative of at least three independent experiments. ***, $P < 0.001$.

cytofluorometry, variations of VLAs (VLA-3 to VLA-6) and $\alpha_v\beta_3$ membrane expression were not observed, either on thymocytes or TEC (data not shown). Nevertheless, laminin-driven migration diminished in Sema-3A-treated thymocytes (Fig. 6*E*), suggesting that the de-adhesion effect of Sema-3A on thymocytes was partially because of the inhibition of VLA binding to laminin.

Discussion

During T cell development, bone marrow-derived precursors migrate toward the thymus. Developing thymocytes travel within the cortex and medulla through mechanisms of chemoattraction (18) and chemorepulsion (19). Appropriate migration is achieved at each thymic compartment where cells undergo positive or negative selection. During this process, there is a requirement of several time-dependent adhesion events of thymocytes to TEC and DC. The cell–cell contact is clearly time dependent but also varies as a

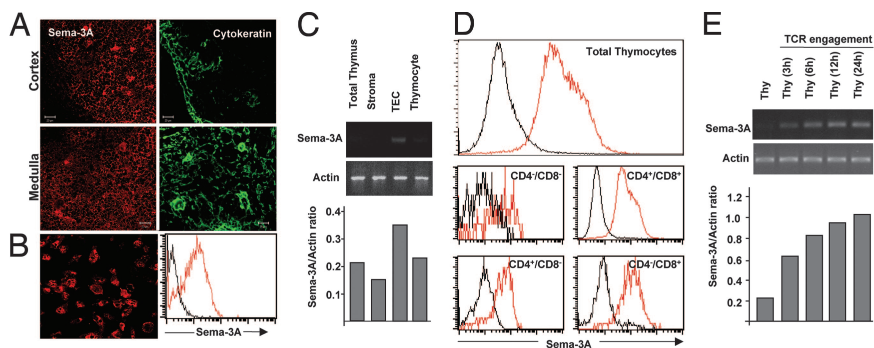

Fig. 4. Sema-3A expression in the human thymus and thymic cell types. (*A*) *In situ* expression of Sema-3A in both cortex and medulla of the thymic lobules. In these double-labeled figures, it is clear that Sema-3A staining comprises the thymic epithelial (cytokeratin-positive) network, but is also seen throughout the tissue, thus including thymocytes. (Original magnification, ×250.) (*B*) The fetal TEC line also expresses Sema-3A as revealed by immunocytochemistry (*Left*) and flow cytometry (*Right*). (Original magnification, ×250.) (*C*) Semiquantitative PCR showing Sema-3A gene expression in the whole thymus, total stroma, TEC, and total thymocytes. Histograms represent the Sema-3A/actin ratio. (*D*) Total and CD4/CD8-defined thymocyte subsets that expressed the Sema-3A (red histograms) compared with irrelevant antibody (black histograms). (*E*) Semiquantitative PCR showing an increase in Sema-3A gene expression on thymocytes following TCR engagement. Histograms represent the Sema-3A/actin ratio. Data shown in each image are representative of at least three independent experiments.

function of the integrin receptor that binds ECM and contributes to the stability of thymocyte–antigen-presenting cell contact (20).

Acquisition of specific membrane proteins in interacting cells represents a means by which thymocytes regulate the strength and time of contact with TEC and DC. In this process, it is conceivable that interactions between receptors and their corresponding ligands may result in impairment and/or termination of adhesion, associated to a migratory effect, secondary to chemoattraction or chemorepulsion. In this respect, the molecules involved in the deadhesion of thymocytes to TEC, as well as in the transmigration of thymocytes toward specific thymic niches, are not well defined.

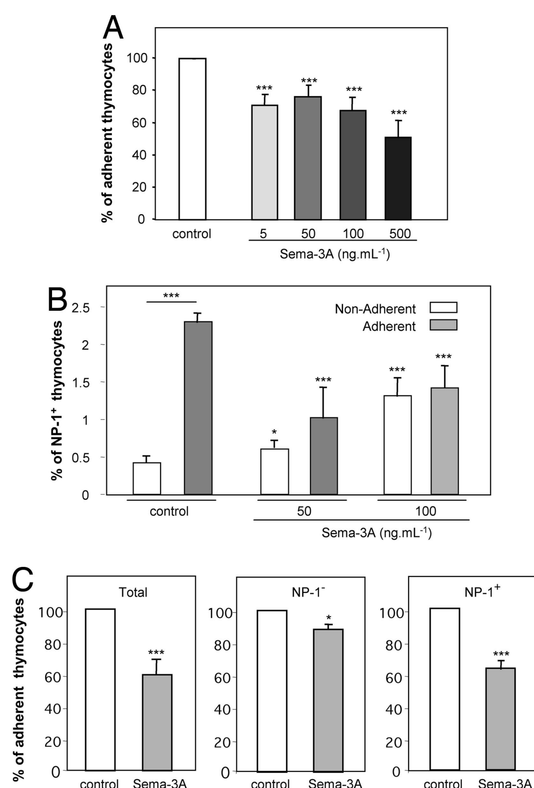

Fig. 5. Sema-3A down-regulates TEC–thymocyte adhesion. (*A*) The recombinant Sema-3A repulsive isoform decreases thymocyte adhesion in a dose-dependent manner (5–500 ng/ml). Control values of untreated cocultures were normalized as 100% of adhesion, and the various Sema-3A values correspond to the means ± SEM of six wells, recovered in three independent experiments. (*B*) Sema-3A (50–100 ng/ml) decreases the relative number of NP-1$^+$-adhered thymocytes (white histograms) and increases NP-1$^+$-nonadherent thymocytes (gray histograms). (*C*) Sema-3A (100 ng/ml) mainly inhibits adhesion of NP-1$^+$ thymocytes, compared with the NP-1-depleted thymocyte fraction. Control values of untreated cocultures in these graphs were normalized to 100% of adhesion. *, $P < 0.05$; ***, $P < 0.001$.

The similarities of interactions formed between interacting antigen-presenting cells and peripheral T cells and the contacts existing between presynaptic and postsynaptic neurons may provide some clues to the understanding of immune regulation within the thymus. We recently demonstrated that NP-1 is expressed by peripheral T cells and DCs, being involved in the interaction between these two cell types (12). Herein, we extend the concept to the T cell development within the human thymus. We found that NP-1 is expressed in both cortex and medulla of thymus, and particularly on all thymocyte CD4/CD8-defined subsets, as well as in TEC (including TNC) and DC. As we have reported, during DC–T cell contact (12), NP-1 is localized and polarized at the site of thymocyte–TEC contact. In addition, up-regulation of NP-1 during this contact prompted us to investigate the putative regulatory role of key intrathymic stimuli, IL-7 secretion and TCR engagement. Both increased NP-1 expression on thymocytes, suggesting an important role of NP-1 during thymocyte–TEC adhesion.

Semaphorins are proteins involved in neuronal axonal guidance and are structurally defined by a 500-aa conserved Sema domain at their N-terminal ending. In axon guidance, the repulsive nature of semaphorin signaling is because of the modification of the growth cone cytoskeleton: the NP-1/Sema-3A signaling mediates the retraction of filopodia and lamellipodia and a localized rearrangement of the actin cytoskeleton (21). It can be hypothesized that this cytoskeleton modification and rearrangement modulates the adhesion capacity of thymocytes, in addition to the repulsive effect of Sema-3A on thymocyte migration. Even though Sema-3A is able to modify thymocyte adhesion and migration, the presence of the NP-1 coreceptor plexin-A is a functional key for this complex.

In particular, plexin-A1 is known to increase the sensitivity and activity of Sema-3A on the actin microfilaments. We have actually detected plexin-A1 on TEC and all thymocyte subsets, indicating a relevant function for the NP-1/Sema-3A complex (data not shown). Interestingly, plexin-A1/Sema-3A signaling induces the association of plexin-A1 to MICAL (22, 23), a CasL interacting molecule, constitutively expressed in thymus (23). Even though the NP-1/Sema-3A complex is functional, being able to influence thymocyte behavior, it remains to be determined the signaling pathway(s) involved in this process and mediated by plexin-A1.

In summary, we show that NP-1 and Sema-3A are constitutively expressed in the human thymus, in both microenvironmental and lymphoid compartments. In thymocytes, NP-1 is up-regulated by IL-7 and TCR engagement, as well as after adhesion to TEC monolayers. Taken together, these data strongly indicate that NP-1 expression on thymocyte subsets is tightly regulated through interactions with the thymic epithelium. Also, we show that heterocel-

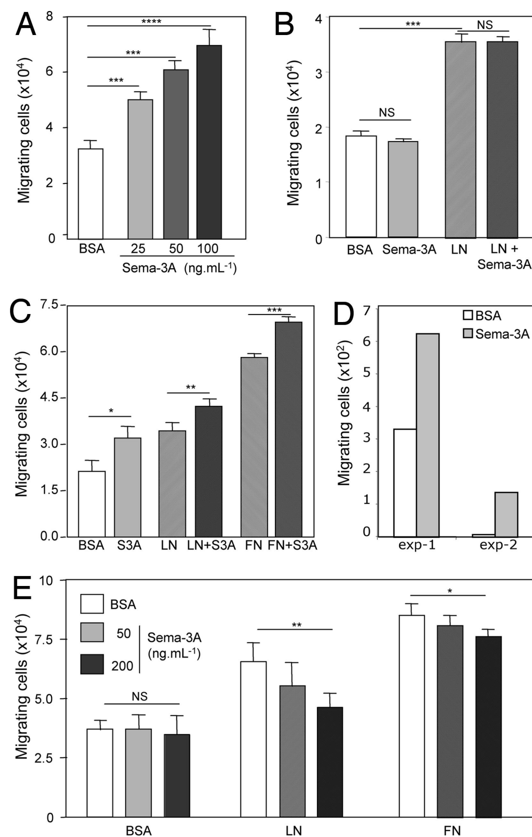

Fig. 6. Chemorepulsive effect of Sema-3A on thymocyte migration. (*A*) Thymocytes were incubated with BSA or Sema-3A (25, 50, or 100 ng/ml) and plated in the upper chamber of Transwell. Histograms represent the number of migrating thymocytes recovered in the lower chamber. (*B*) Sema-3A and laminin (LN) were plated in the lower chamber and insert, respectively, to test the chemoattractant property of these proteins on thymocytes. Histograms represent the number of migrating thymocytes in the lower chamber. (*C*) In presence of BSA or Sema-3A (100 ng/ml), thymocytes were plated in the upper chamber of Transwell under chemoattraction driven by laminin (LN) or fibronectin (FN). Histograms represent the number of migrating thymocytes in the lower chamber. (*D*) Thymocytes were incubated in the presence of BSA or Sema-3A (100 ng/ml) and plated in the first well of horizontal migration slide. Histograms show the number of migrating thymocytes toward the opposite side of two independent experiments. (*E*) Thymocytes were incubated with BSA or Sema-3A (50 or 200 ng/ml) and plated in the upper chamber of a Transwell coated with laminin or fibronectin. Histograms represent the number of migrating thymocytes in the lower chamber. Histograms represent the mean ± SEM of at least four wells, evaluated in at least two independent experiments. NS, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

lular interactions between thymocytes and TEC are partially dependent on NP-1/Sema-3A-mediated interactions, and that Sema-3A exerts a de-adhesive action on this contact. Moreover, Sema-3A/NP-1 ligation modulates thymocyte migration acting as a chemorepulsive stimulus.

Lastly, our data reinforce the notion that human thymocyte migration is a complex event, comprising multiple molecular interactions, leading to chemoattraction and chemorepulsion, that are driven by a variety of secreted moieties, including at least ECM proteins, chemokines, and (as shown in this article) semaphorins. These results lead us to propose the following hypothetical model. During maturation, thymocytes interact with various cell types (such as TEC) through an adhesion → deadhesion mechanism that plays a crucial role in thymocyte differentiation. In this respect, our findings favor the implication of NP-1 and Sema-3A in the regulation of events contributing to thymocyte migration and adhesion. The first contact between TEC and thymocyte depends on NP-1 up-regulation mediated by TEC-derived IL-7, and this event enhances focal adhesion of thymocytes. In a second vein, TCR engagement will induce expression of both NP-1 and Sema-3A. Increased NP-1 expression will increase sensitivity of thymocytes to Sema-3A. Secreted Sema-3A will in turn form a complex with NP-1, triggering signaling through the NP-1 coreceptor, plexin-A1. This multiple complex will then induce de-adhesion and chemorepulsion of thymocytes, thus being involved in their normal guidance. These events highlight the importance of NP-1/Sema-3A complex in the regulation of migration and adhesion of human thymocytes.

Materials and Methods

Antibodies and Chemicals. Monoclonal antibodies (mAbs) used in flow cytometry were BDCA-4-phycoerythrin (PE) or -allophycocyanin (anti-NP-1) (Miltenyi Biotec, Bergisch Gladbach, Germany), anti-VLA-3 to -6-FITC (Cymbus, Southampton, U.K.), anti-$\alpha_v\beta_3$-FITC (Chemicon, Temecula, CA), anti-CD8-FITC, -PE or -allophycocyanin, anti-CD4-FITC or -PE-Cy5, anti-CD11b-PE, anti-CD11c-PE, anti-CD13-PE-Cy5, and appropriate Ig isotype controls (Immunotech, Marseille, France) and anti-CD123w-PE (Becton Dickinson). Recombinant human Fc-Sema-3A, IL-7, as well as anti-TCR (UCH-T1) were purchased from R&D Systems (Lille, France). Anti-Sema-3A mAb was kindly provided by A. Shirvan Felsenstein Medical Research Center, Petach-Tiava, Israel (24) and was revealed by using a goat anti-mouse-Ig-PE (Beckman Coulter, Villepinte, France). The antibodies used in immunohistochemistry were anti-cytokeratin mAb KL-1 (Immunotech) and anti-NP-1 polyclonal antibody (12) revealed by FITC-labeled goat anti-mouse IgG and Cy5-labeled goat anti-rabbit IgG second antibody, respectively (Jackson ImmunoResearch, West Grove, PA).

Thymus Samples and TEC Cultures. Human thymic tissue was obtained from children undergoing cardiac surgery (n = 19), aged from 1 day to 9 years. Experimental procedures with human thymic fragments have been approved by the Oswaldo Cruz Foundation and the Necker Hospital Ethical Committees for human research, and were done according to the European Union guidelines and the Declaration of Helsinki.

Some fragments were snap frozen in liquid nitrogen and used for *in situ* immunohistochemistry. Other fragments were processed for isolation of TNC. In culture, TNC spontaneously release thymocytes, so that after 3–5 days in culture, we had a TNC-derived epithelial monolayer (25). TNC were isolated from human thymic fragments as currently done in our laboratory (26), and cytofluorometric detection of NP-1 was done on lymphoepithelial complexes or after obtaining highly purified TNC-derived epithelial cultures. Because in TNC isolation, nonepithelial contaminants may be present, the cytofluorometric analysis was done after double-staining with an anti-cytokeratin antibody (Dako, Carpinteria, CA), so that we first gated cytokeratin-positive cells, and then ascertained NP-1 in this gate.

Two human TEC lines were obtained by an explant technique and limiting dilution cloning, being kindly provided by Dr. M. L. Toribio (Universidad Autonoma de Madrid, Madrid, Spain). One TEC line was derived from a fetal thymus, whereas the other was obtained from an infant thymus (27).

Immunofluorescence and Cytofluorometry. Thymus frozen sections and TEC cultures were double labeled according to routine procedures. Specimens were subjected to anti-NP-1 antibody followed by rhodamine-labeled goat anti-rabbit IgG. Second, anti-cytokeratin mAb was revealed by FITC-labeled goat anti-mouse IgG. Analysis was done by confocal microscopy. TEC and thymocytes plated on poly-L-lysine-coated (Sigma–Aldrich, Lyon, France) slides were fixed in 4% paraformaldehyde, quenched with 0.1 M glycine, and permeabilized with 1% saponin. Cells were incubated with the anti-Sema-3A, anti-NP-1, or anti-cytokeratin mAb. Pri-

mary antibody was revealed by a goat anti-mouse or -rabbit IgG conjugated to Cy-5, respectively. Specimens were examined with a confocal laser microscope (LSM 510; Carl Zeiss, Oberkochen, Germany).

For flow cytometry, cells were fixed in 4% paraformaldehyde and incubated for 15 min at 4°C in PBS, 2% BSA, containing 0.05% saponin, with anti-Sema-3A or control isotype-matched irrelevant antibodies. Specific labeling was revealed by using a goat anti-mouse-Ig-PE. Membrane staining was performed by using FITC- and allophycocyanin-conjugated anti-CD4, anti-CD8 antibodies. Specimens were analyzed with a FACSCalibur (Becton Dickinson). In some experiments, four-color labeling was done to detect NP-1 in DCs. In this case, we performed simultaneous labeling for NP-1, CD4, CD8, and a given DC marker, CD11c or CD13.

RNA Isolation and Semiquantitative RT-PCR. Total RNA was extracted from 10^7 total thymus, total stroma, TEC, unstimulated and IL-7- or TCR-stimulated thymocytes, at different stages, and reverse transcribed by using the first-strand cDNA synthesis kit according to the manufacturer's instructions (Amersham Biosciences, Les Ulis, France). The PCR was carried out with cDNA as template, 50 pmol of both primers in the reaction mixture [50 mM KCl, 10 mM Tris·HCl, pH 8.3, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 0.4 mM each dNTP (Promega, Madison, WI), 2.5 units of AmpliTaq polymerase (Applied Biosystems, Foster City, CA)]. After denaturation at 94°C for 5 min, samples underwent 25 amplification cycles. Sense and antisense primers were as follows, respectively: 5′-CTAGAAGCATTTGCGGTGGACGATGGAGGG-3′ and 5′-TGACGGGGTCACCCACACTGTGCCCATCTA-3′ for β-actin; 5′-ACTCACTGTTCAGACTTA-3′ and 5′-AGAGACTTCATGCAGCTC-3′ for Sema-3A; and 5′-CTGGTGAGCCCTGTGGTTTATTCC-3′ and 5′-ACTATTGTCATCCACAGCAATCCC-3′ for NP-1. PCR products were analyzed on 1.5% agarose gel. Semiquantitative analysis was performed by the Gel Doc 2000 System (Bio-Rad, Hercules, CA). Sema-3A or NP-1 cDNA was normalized to β-actin cDNA concentration, and Sema-3A/β-actin or NP-1/β-actin cDNA ratios were calculated.

Thymocyte Stimulation. Freshly isolated thymocytes were cultured in presence of IL-7 (10 ng/ml) during 6–12 h. Then, cells were harvested and cytofluorometry analysis of NP-1 expression or mRNA extractions were performed to follow both NP-1 and Sema-3A protein and mRNA regulation, respectively. To induce TCR engagement, thymocytes were plated in the presence of coated anti-TCR antibody (UCHT-1; 5 μg/ml) or uncoated wells during 3–24 h at 37°C. Thymocytes were harvested at different times and stained with mAbs to follow NP-1 expression, or used to extract mRNA to detect NP-1 and Sema-3A mRNA by semiquantitative RT-PCR.

Cell Adhesion. Human TEC cultures were harvested, and 4×10^5 cells were replated in 75 cm^2 flasks (Nunc, Roskilde, Denmark). After 24 h, thymocytes were cocultured (50 thymocytes per TEC) for 2 h at 37°C. Adherent cells were counted and phenotyped by cytofluorometry. We tested the inhibitory effect of Sema-3A on thymocyte adhesion by preincubating thymocytes with various concentrations of the protein (5–500 ng/ml), or the medium alone, for 2 h at 37°C. Nonadherent cells were gently washed out, and adhered thymocytes were counted and phenotyped.

Chemotaxis Assay. Thymocyte migratory activity was assessed *ex vivo* in 5-μm pore size Transwell plates (Corning Costar, Corning, NY), as previously reported (28). Membrane inserts were coated with BSA, laminin, or fibronectin for 1 h at 37°C, followed by 1 h of blocking with 1% BSA. Thymocytes (2.5×10^6) with Sema-3A (25–100 ng/ml) were plated in the upper chamber in 100 μl of 0.5% BSA/RPMI, and 600 μl of 0.5% BSA/RPMI were added to the lower chamber. After 3 h, cells that migrated into lower chambers were removed, counted, and phenotyped for the detection of NP-1, CD4, and CD8. In some experiments, Sema-3A was placed in the bottom well of the Transwell chamber to test a possible chemoattractant effect of the molecule.

In the horizontal migration assay (Biovalley, Conches, France), cells were plated in the first chamber side in presence of BSA or Sema-3A (100 ng/ml). After 30–60 min, we counted those cells migrating toward the opposite chamber through a capillary ridge.

Purification of NP-1$^+$ Thymocytes. Total thymocytes (10^9 cells) were incubated during 30 min at 4°C in presence of allophycocyanin-coupled anti-NP-1 mAb, washed in 2% FCS/PBS solution, and incubated with anti-allophycocyanine magnetic beads during 30 min at 4°C before being plated in a magnetic sorter (Miltenyi Biotec). NP-1$^-$ thymocytes were directly eluted, whereas NP-1$^+$ cells were retained in the column. Then, total, NP-1$^-$, and NP-1$^+$ thymocytes were used in culture to test the effect of Sema-3A (100 ng/ml) on thymocyte adhesion to TEC monolayers.

Statistical Analysis. The results were analyzed by independent sample two-tailed and unpaired Student's *t* test, and were presented as means ± standard error.

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1. Ansel KM, Cyster JG (2001) *Curr Opin Immunol* 13:172–179.
2. Annunziato F, Romagnani P, Cosmi L, Lazzeri E, Romagnani S (2001) *Trends Immunol* 22:277–281.
3. Savino W, Mendes-da-Cruz DA, Silva JS, Dardenne M, Cotta-de-Almeida V (2002) *Trends Immunol* 23:305–313.
4. Savino W, Mendes-Da-Cruz DA, Smaniotto S, Silva-Monteiro E, Villa-Verde DM (2004) *J Leukoc Biol* 75:951–961.
5. Steinman L (2004) *Nat Immunol* 5:575–581.
6. Limatola C, Di Bartolomeo S, Trettel F, Lauro C, Ciotti MT, Mercanti D, Castellani L, Eusebi F (2003) *J Neuroimmunol* 134:61–71.
7. Sun L, Lee J, Fine HA (2004) *J Clin Invest* 113:1364–1374.
8. He Z, Tessier-Lavigne M (1997) *Cell* 90:739–751.
9. Kolodkin AL, Levengood DV, Rowe EG, Tai YT, Giger RJ, Ginty DD (1997) *Cell* 90:753–762.
10. Fan J, Mansfield SG, Redmond T, Gordon-Weeks PR, Raper JA (1993) *J Cell Biol* 121:867–878.
11. Miao HQ, Soker S, Feiner L, Alonso JL, Raper JA, Klagsbrun M (1999) *J Cell Biol* 146:233–242.
12. Tordjman R, Lepelletier Y, Lemarchandel V, Cambot M, Gaulard P, Hermine O, Romeo PH (2002) *Nat Immunol* 3:477–482.
13. Serini G, Valdembri D, Zanivan S, Morterra G, Burkhardt C, Caccavari F, Zammataro L, Primo L, Tamagnone L, Logan M, *et al.* (2003) *Nature* 424:391–397.
14. Vandenabeele S, Hochrein H, Mavaddat N, Winkel K, Shortman K (2001) *Blood* 97:1733–1741.
15. Bendriss-Vermare N, Barthelemy C, Durand I, Bruand C, Dezutter-Dambuyant C, Moulian N, Berrih-Aknin S, Caux C, Trinchieri G, Briere F (2001) *J Clin Invest* 107:835–844.
16. Offner F, Plum J (1998) *Leuk Lymphoma* 30:87–99.
17. Schwinzer R, Sommermeyer H, Schlitt HJ, Schmidt RE, Wonigeit K (1991) *Cell Immunol* 136:318–328.
18. Kim CH, Pelus LM, White JR, Broxmeyer HE (1998) *Blood* 91:4434–4443.
19. Cyster JG (2002) *J Clin Invest* 109:1011–1012.
20. Ayres-Martins S, Lannes-Vieira J, Farias-De-Oliveira DA, Brito JM, Villa-Verde DM, Savino W (2004) *Cell Immunol* 229:21–30.
21. Fournier AE, Nakamura F, Kawamoto S, Goshima Y, Kalb RG, Strittmatter SM (2000) *J Cell Biol* 149:411–422.
22. Terman JR, Mao T, Pasterkamp RJ, Yu HH, Kolodkin AL (2002) *Cell* 109:887–900.
23. Suzuki T, Nakamoto T, Ogawa S, Seo S, Matsumura T, Tachibana K, Morimoto C, Hirai H (2002) *J Biol Chem* 277:14933–14941.
24. Shirvan A, Ziv I, Fleminger G, Shina R, He Z, Brudo I, Melamed E, Barzilai A (1999) *J Neurochem* 73:961–971.
25. Guyden JC, Pezzano M (2003) *Int Rev Cytol* 223:1–37.
26. Ribeiro-Carvalho MM, Farias-de-Oliveira DA, Villa-Verde DM, Savino W (2002) *Neuroimmunomodulation* 10:142–152.
27. Fernandez E, Vicente A, Zapata A, Brera B, Lozano JJ, Martinez C, Toribio ML (1994) *Blood* 83:3245–3254.
28. Cotta-de-Almeida V, Villa-Verde DM, Lepault F, Pleau JM, Dardenne M, Savino W (2004) *Eur J Immunol* 34:1578–1587.