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Semaphorin-3A-Related Reduction of Thymocyte Migration in Chemically Induced Diabetic Mice

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Keywords
Thymus · Semaphorin · Atrophy · Diabetes · Thymocyte migration

Abstract

Background: Previous work revealed the existence of a severe thymic atrophy with massive loss of immature $CD4^+CD8^+$ thymocytes in animals developing insulin-dependent diabetes, chemically induced by alloxan. Furthermore, the intrathymic expression of chemokines, such as CXCL12, is changed in these animals, suggesting that cell migration-related patterns may be altered. One molecular interaction involved in normal thymocyte migration is that mediated by soluble semaphorin-3A and its cognate receptor neuropilin-1. ***Objectives:*** We investigated herein the expression and role of semaphorin-3A in the migratory responses of thymocytes from alloxan-induced diabetic mice. We characterized semaphorin-3A and its receptor, neuropilin-1, in thymuses from control and diabetic mice as well as semaphorin-3A-dependent migration of developing thymocytes in both control and diabetic animals. ***Methods:*** Diabetes was chemically induced after a single injection of alloxan in young adult BALB/c mice. Thymocytes were excised from control and diabetic individuals and subjected to cytofluorometry for simultaneous detection of semaphorin-3A or neuropilin-1 in CD4/CD8-defined subsets. Cell migration in response to semaphorin-3A was performed using cell migration transwell chambers. ***Results:*** Confirming previous data, we observed a severe decrease in the total numbers of thymocytes in diabetic mice, which comprised alterations in both immature (double-negative subpopulations) and mature CD4/CD8-defined thymocyte subsets. These were accompanied by a decrease in the absolute numbers of semaphorin-3A-bearing thymocytes, comprising $CD4^-CD8^-$, $CD4^+CD8^+$, and $CD4^-CD8^+$ cells. Additionally, immature $CD4^-CD8^-$ and $CD4^+CD8^+$ developing T cells exhibited a decrease in the membrane density of semaphorin-3A. The relative and absolute numbers of neuropilin-1-positive thymocytes were also decreased in diabetic mouse thymocytes compared to controls, as seen in $CD4^-CD8^-$, $CD4^+CD8^+$, and $CD4^-CD8^+$ cell subpopulations. Functionally, we observed a decrease in the

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chemorepulsive role of semaphorin-3A, as revealed by transwell migration chambers. Such an effect was seen in all immature and mature thymocyte subsets. ***Conclusions:*** Taken together, our data clearly unravel a disruption in the normal cell migration pattern of developing thymocytes following chemically induced insulin-dependent diabetes, as ascertained by the altered migratory response to sempahorin-3A. In conceptual terms, it is plausible to think that such disturbances in the migration pattern of thymocytes from these diabetic animals may exert an impact in the cell-mediated immune response of these mice.

Introduction

T lymphocytes originate from hematopoietic stem cells in bone marrow that populate the thymus as early thymic progenitors (ETP), which expand and differentiate in thymocyte subpopulations [1–3]. Thymocyte differentiation is coordinated by several signals from the thymic microenvironment, being associated with precisely oriented migration across the thymic lobules [4].

Thymocyte migration is controlled by a group of molecular interactions comprising, among others, those involving chemokines, extracellular matrix, sphingolipids, and hormones [2, 5]. We have previously postulated that the oriented migration of these cells can be conceived as a multivectorial event, so that each ligand-receptor interaction corresponds to one given vector, the cell displacement being the result of the multiple interactions, thus corresponding to the resulting vector [6]. Moreover, one given interaction can influence another, positively or negatively, as for example, there is a synergy of CXCL12 and laminin in generating a migratory response of thymocytes [7]. In addition, among cell migration-related interactions in the thymus, there are attractant as well as repulsive stimuli [8].

We previously demonstrated that semaphorin-3A (SEMA-3A) exerts a chemorepulsive effect upon human thymocytes, being also able to partially impair the chemoattractant effects of laminin and CXCL12 [9–11]. Additionally, we showed that both human developing thymocytes and thymic epithelial cells constitutively express SEMA-3A. These cell types also express the cognate SEMA-3A receptor, neuropilin-1 (NPR-1) as well as plexin-A and plexin-B, which are able to transduce intracellularly the SEMA-3A stimulus sensed by NPR-1 [9–11]. Interestingly, similar effects in the human thymus were seen with another member of the semaphorin family, namely SEMA-3F, which acts through the receptor neuropilin-2 [12].

Although the physiological role of SEMA-3A/NPR-1 in the thymus has been partially established, literature concerning changes of this ligand-receptor in pathological conditions remains largely unknown.

The chemically induced diabetic mice have been used as a model for therapeutic and preventive studies from the biochemistry to the morphologic alterations that can occur during and after the diabetic condition [13].

We previously reported that, in the chemically induced diabetic mice, there is a thymic involution, with a decrease in the expression of CXCL12 and its receptor CXCR4 by thymocytes, accompanied by a diminution in total thymocyte numbers; and those results are not from the alloxan toxicity [12, 14]. This was not due to a direct effect of the chemical applied (alloxan), since we also showed that it has no cytotoxic effect. Accordingly, the thymic alterations observed in the chemically induced diabetic mice are due to the effects of high blood glycemia and hormone disturbance but not by direct cytotoxicity of alloxan to developing thymocytes [14]. In a second vein, it was shown that SEMA-3A is downregulated in the bone of streptozotocin-induced diabetic rats [15].

Thus, given that SEMA-3A is involved in thymocyte de-adhesion to microenvironmental cells, partially inhibits thymocyte migration towards CXCL12, and is downregulated in the bone of chemically induced diabetic rats, we raised the hypothesis that the intrathymic SEMA-3A/NRP-1 expression patterns might be altered in the thymus of such animals, with consequent changes upon thymocyte migration.

Materials and Methods

Animals and Diabetes Induction

Specific pathogen-free BALB/c male mice, aged 6–8 weeks, were obtained from the animal facility at the University of Campinas (Centro Multi-Institucional de Bioterismo, Campinas, Brazil). The animals were maintained in transparent acrylic plastic isolators under aseptic conditions throughout the study. Sterile water and feed were provided ad libitum.

For chemical induction of diabetes, 10 animals were given a single injection of alloxan (180 mg/kg) diluted in citrate buffer 0.1 M pH 6.0 intraperitoneally. A control group of 3 animals were given a single dose of citrate buffer 0.1 M. After 48 h of drug injection, blood glucose levels were assessed and only animals with glucose levels >220 mg/dL were considered diabetic and used in the experiments.

Flow Cytometry

Thymocytes were obtained from freshly isolated control and diabetic mouse thymuses. Once in cell suspension, thymocytes were washed and resuspended in PBS 5% fetal calf serum for subsequent cell counting and staining to directly conjugated CD3-PECY7, CD4-

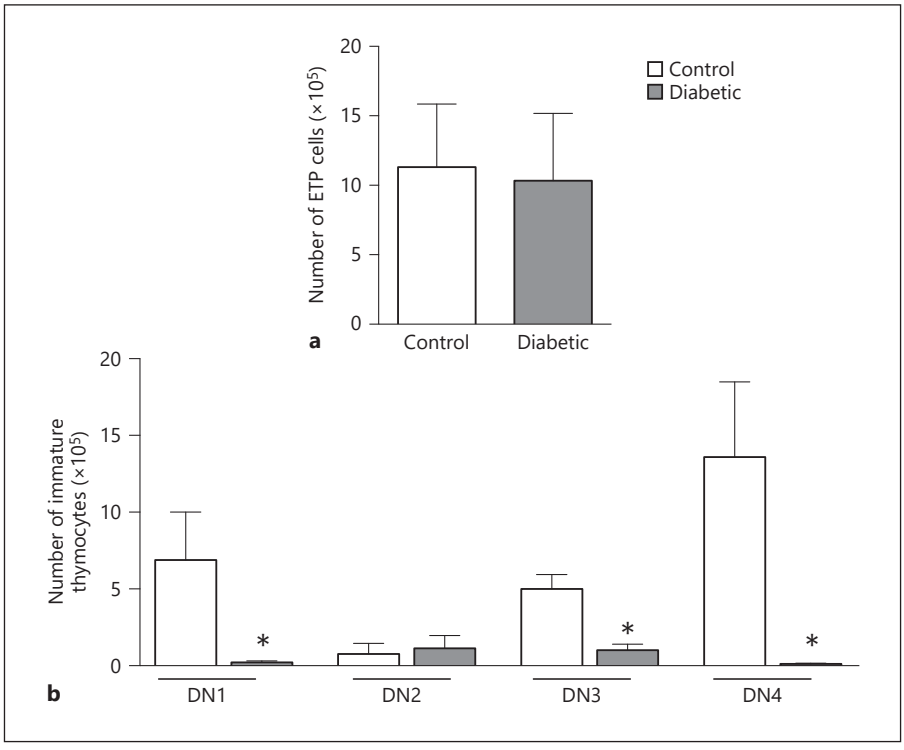

Fig. 1. Decrease in thymus cellularity in chemically induced diabetic mice. Compared to control animals, thymuses from diabetic mice presented alterations in the proportion of developing thymocyte subpopulations (**a**) as well a reduction in the total number of thymocytes and in CD4/CD8-defined subpopulations (**b**). Results are expressed as mean ± SE for at least 5 animals. ** $p \leq 0.01$.

FITC, CD8-APC, NRP-1-PERCPCy5 and SEMA-3A-PE, CD3-PECY7, CD4- PERCPCy5, CD8-APC, CD44-FITC and CD8-PE (Mix 2) or Lineage-PE, CD44-FITC and c-Kit (APC) monoclonal antibodies. For the ETP cell analysis a magnetic negative separation to $CD4^+$ was performed in order to enrich the cell population to immature thymocytes. The splenocytes were obtained from control and diabetic spleens, which were processed to red blood cell lysis and subsequent cell counting and staining to directly conjugated CD4-FITC, CD8-APC, CD62L-PERCPCy5, and CD44-PE monoclonal antibodies. The antibodies, at the appropriated concentrations, were added to the cells and incubated for 20 min on ice in the dark. After immunostaining, cells (both thymocytes and splenocytes) were fixed and analyzed by flow cytometry in FACS Canto II device (Becton Dickinson, San Jose, CA, USA).

Transmigration Assay

Thymocyte migratory response to SEMA-3A was assessed ex vivo in 5-μm pore-size transwell plates (Corning Costar, Cambridge, MA, USA) using cell suspension after pooling three thymuses from diabetic or control animals, as previously described [14]. Briefly, membranes were blocked with 1% BSA-RPMI 1640 for 1 h and thymocytes (2.5 million cells) were added to the upper chamber in 100 μL of 0.5% BSA-RPMI 1640 with or without SEMA-3A (50 ng/mL), and 600 μL of 0.5% BSA-RPMI 1640 were added in the lower chamber. Alternatively, we preincubated thymocytes with SEMA-3A for 30 min, washed them, and then added them to the upper chamber. In all cases, after 3 h of incubation at 37 °C in a 5% CO_2 humidified atmosphere, migrating cells were ultimately counted, labeled with appropriate antibodies, and analyzed by flow cytometry. The specific migration, driven by SEMA-3A, was expressed after subtracting the value recovered in wells containing only BSA.

Statistical Evaluation

Statistical evaluation of the results was done by using the unpaired Student *t* and Kruskal-Wallis tests. The GraphPad Instat software version 4.0 (GraphPad, La Jolla, CA, USA) was applied for these analyses. Differences between groups were considered statistically significant when *p* values were <0.05.

Results

We first analyzed thymocyte subpopulations to evaluate the alloxan-induced thymus atrophy (Fig. 1). Indeed, we observed alterations in thymocyte populations from diabetic mice with severe loss of total thymocyte numbers, in particular $CD4^+CD8^+$ subsets (Fig. 1b), as observed by our group previously [14]. However, we found some differences, likely due to the fact that we worked with a mouse cohort under high blood glycemia (data not shown). Since diminution of double-positive cells was more important, we could see the relative increase in the percentages of the other subpopulations (Fig. 1a).

Since the thymus of chemically induced diabetic animals revealed the expected thymic atrophy, we analyzed the early progenitor cells (ETP) and double-negative (DN) subpopulations (Fig. 2). We showed that in conditions of high blood glycemia (>600 mg/dL) for 24 h, we

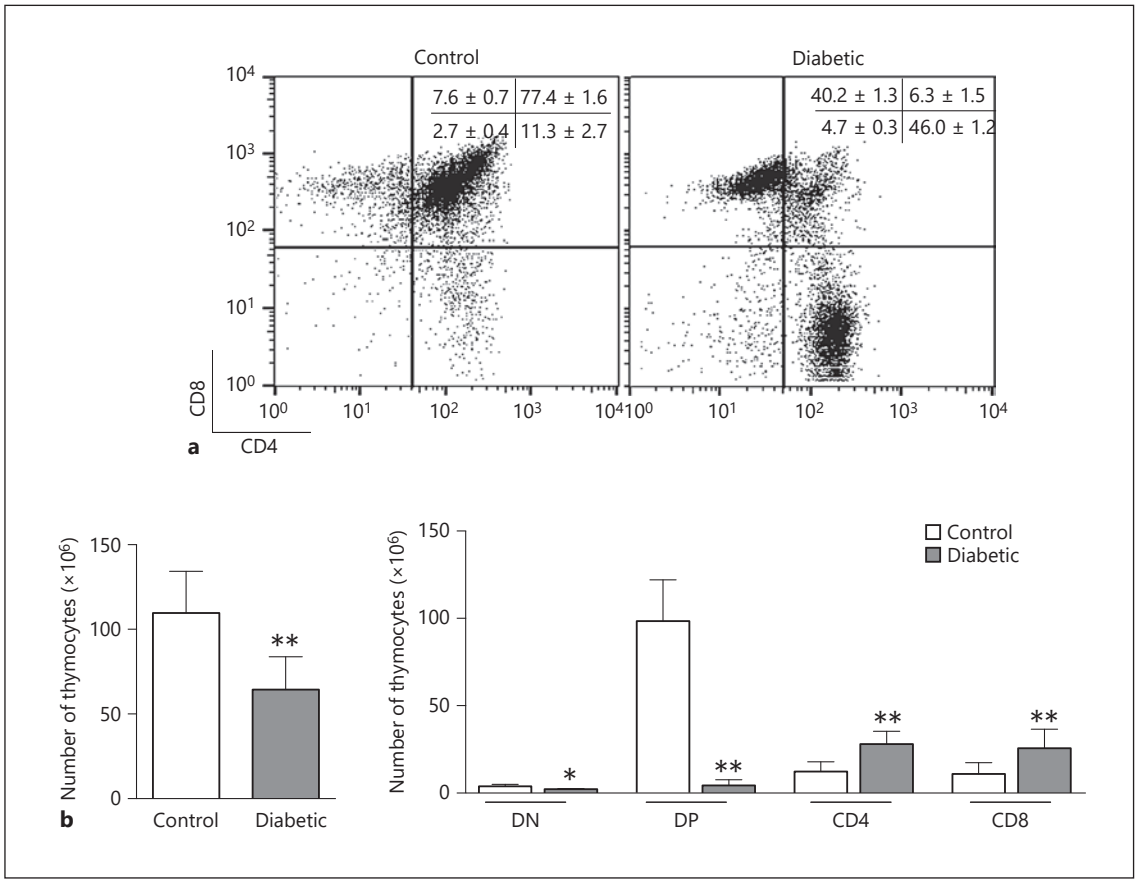

Fig. 2. Changes in the patterns of $CD4^{-}CD8^{-}$ subsets in chemically induced diabetic mice. Although we did not find alterations in the early progenitor cell numbers in diabetic compared to healthy control mice (**a**), we found a significant reduction in the numbers of DN1, DN3, and DN4 (**b**) in the thymuses of diabetic mice compared to controls. Results are expressed as mean ± SE for at least 5 animals. * $p \leq 0.05$.

did not find any alteration in the absolute number of ETP cells (Fig. 2a). Differently, we did observe changes in the numbers of DN1, DN3, and DN4 subpopulations, which were decreased in diabetic mice when compared to the control healthy animals (Fig. 2b). No alteration was observed in the DN2 population.

Next, we evaluated the expression of SEMA-3A in thymocytes, using flow cytometry (Fig. 3). We did not find any significant changes in the relative numbers of $SEMA\text{-}3A^{+}$ cells within thymocyte subsets (Fig. 3a–c). Nevertheless, we observed a decrease in the absolute numbers of all CD4/CD8-defined subpopulations of $SEMA3A^{+}$ thymocytes in diabetic mice compared to controls (Fig. 3d, e). Interestingly, further analysis of SEMA-3A expression also revealed a decrease in the median fluorescence intensity, seen in diabetic mice compared to controls, in both total thymocyte numbers and immature $CD4^{-}CD8^{-}$ and $CD4^{+}CD8^{+}$ subpopulations (Fig. 3f–h).

We also analyzed the membrane expression of the SEMA-3A receptor, NRP-1, by cytofluorometry (Fig. 4). As expected, there was a strong reduction in the relative and total numbers of $NPR\text{-}1^{+}$ thymocytes in diabetic mice compared to controls (Fig. 4a–d). This was also seen in CD4/CD8-defined subsets, with the exception of mature $CD4^{+}$ single positive cells (Fig. 4e). When we considered the median fluorescence intensity of NRP-1 labelling in thymocyte subpopulations, again we showed a significant decrease in all subsets but the mature $CD4^{+}$ single positive cells (Fig. 4f–h).

Given that SEMA-3A/NPR-1 interaction is involved in thymocyte migration, together with the observed changes in the expression pattern of these molecules in the thymus of chemically induced diabetic mice, we analyzed the functional impact of these alterations in terms of the ex vivo thymocyte migratory response to SEMA-3A (Fig. 5). For that we used two experimental conditions: (a) pre-

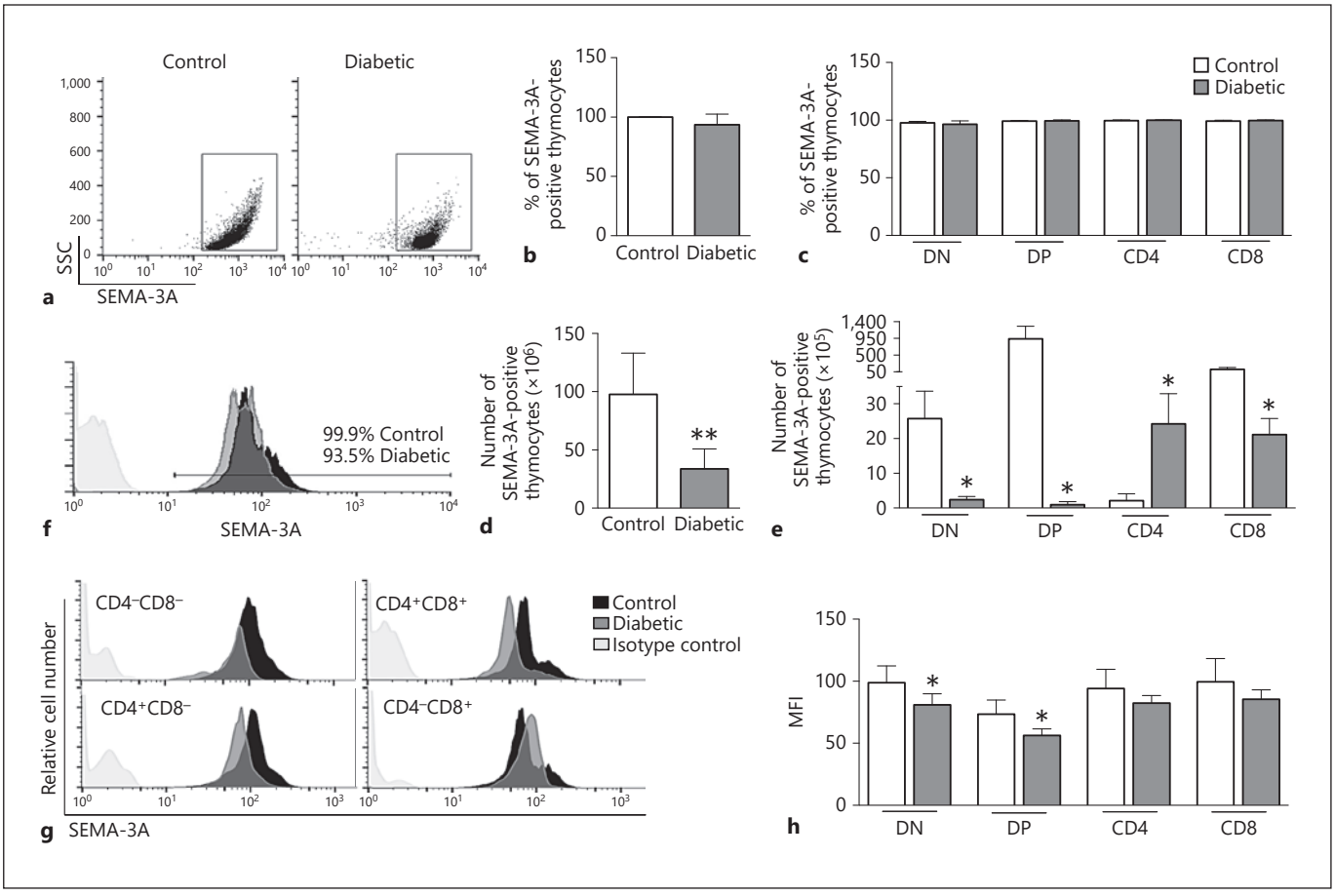

Fig. 3. Decrease of semaphorin-3A (SEMA-3A) expression in thymocytes from chemically induced diabetic mice. **a** Relative number of SEMA-3A$^+$ in total thymocytes. Percentages (**b**, **c**) and median fluorescence intensity (MFI) (**d**, **e**) of SEMA-3A$^+$ in each CD4/CD8-defined thymocyte subset. **f–h** Analysis of SEMA3A membrane density in thymocytes from control or diabetic mice. Typical flow cytometry profiles of SEMA-3A labeling can be seen in **f** and **g**, in control (black histograms) and diabetic thymocyte subsets (grey histograms). The light grey curve corresponds to the labeling generated by an irrelevant fluorochrome-matched IgG. **h** Quantitation of SEMA-3A MFI in thymocyte subpopulations from control and diabetic thymuses. Results are expressed as mean ± SE, using at least 5 animals from each group. * $p \leq 0.05$ and ** $p \leq 0.01$.

incubation of thymocytes plus washing and then allowing them to migrate across the porous membranes of the transwell system, and (b) migration in the presence of SEMA-3A in the supernatants, without any washing procedure. As expected, when compared to BSA, thymocytes from control mice presented an increase in the chemorepulsive migratory response to SEMA-3A when this molecule was added in the upper compartment of the transwell chambers. By contrast, in both experimental conditions, thymocytes from diabetic mice exhibited reduced chemorepulsive migratory response specific to SEMA-3A, when compared to controls (Fig. 5a). Such reduction was seen in all CD4/CD8-defined subsets (Fig. 5b).

Taking into account the findings described above, it seemed conceivable that thymocyte egress from the thymus of chemically induced diabetic mice could be compromised with consequences on the numbers of T cells in the periphery of the immune system. We approached this issue by identifying the numbers of naïve T lymphocytes in the spleen, as indirect evidence for putative alterations in thymocyte export (Fig. 6). Indeed, we observed a reduction in the total numbers of splenocytes (Fig. 6a), including both naïve CD4$^+$ and CD8$^+$ T lymphocytes (Fig. 6b). Of note, such decrease comprised naïve T cells bearing the phenotype CD4$^+$CD62L$^+$CD44$^-$ and CD8$^+$CD62L$^+$CD44$^-$.

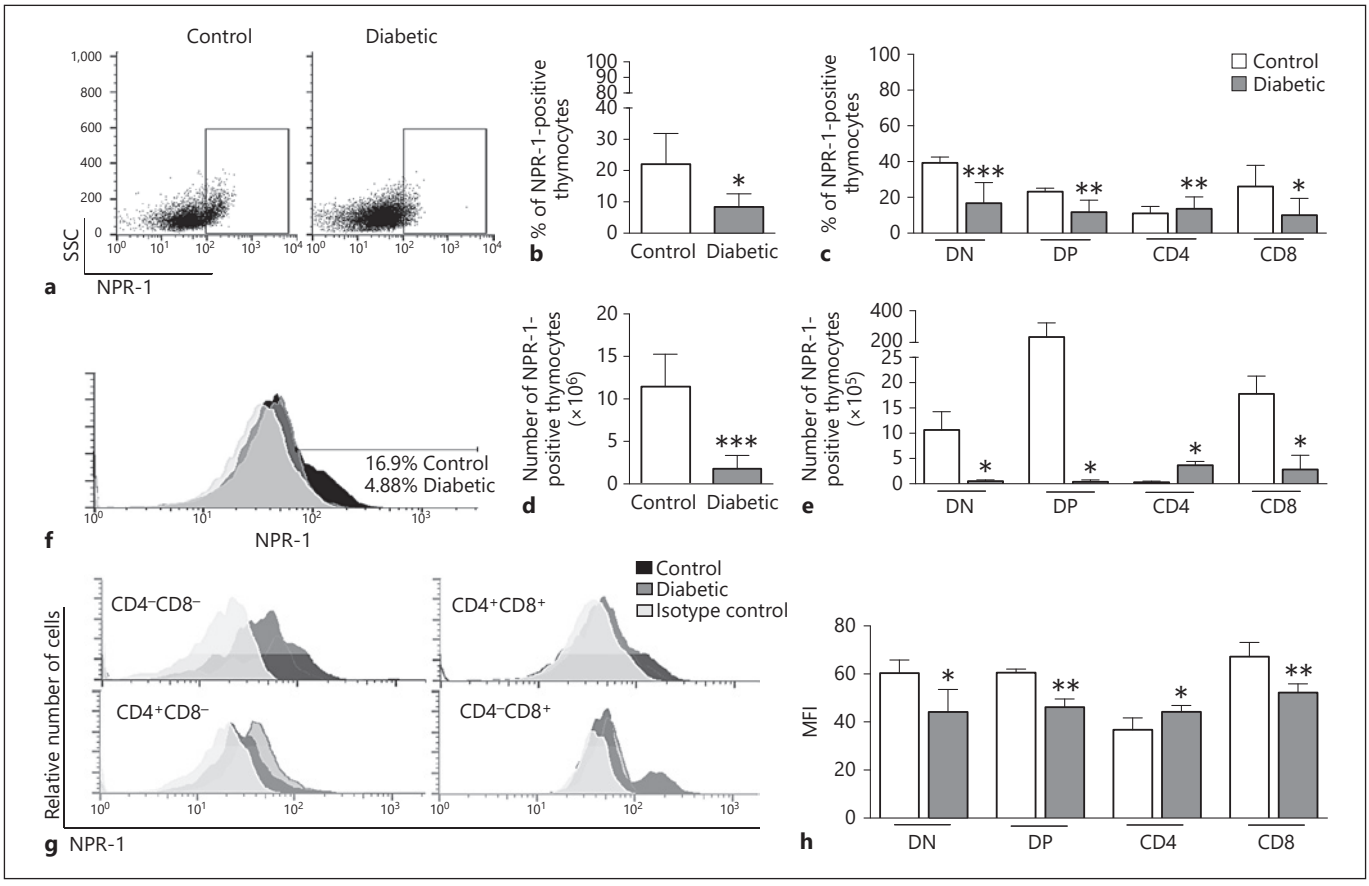

Fig. 4. Decrease of neuropilin-1 (NPR-1) expression in thymocytes from chemically induced diabetic mice. **a** Relative numbers of NPR-1$^+$ in total thymocytes. Percentages (**b**, **c**) and median fluorescence intensity (MFI) (**d**, **e**) of SEMA-3A$^+$ in each CD4/CD8-defined thymocyte subset. **f–h** Analysis of NPR-1 membrane density in thymocytes from control or diabetic mice. Typical flow cytometry profiles of NPR-1 labeling can be seen in **f** and **g**, in control (black histograms) and diabetic thymocyte subsets (grey histograms). The light grey curve corresponds to the labeling generated by an irrelevant fluorochrome-matched IgG. **h** Quantitation of NPR-1 MFI in thymocyte subpopulations from control and diabetic thymuses. Results are expressed as mean ± SE, using at least 5 animals from each group. * $p \leq 0.05$, ** $p \leq 0.01$, and *** $p \leq 0.001$.

Discussion

Class III semaphorins are found in vertebrates, being secreted proteins with structural conservation of the SEMA domain. The amino acids presented in the SEMA domain appear to be responsible by the biological specificity of semaphorin molecules, and only the class III lacks a cell-surface attachment point [16]. Once secreted, its carboxy-terminal tail binds the semaphorin molecule to the extracellular matrix elements of a given tissue [17]. NPR-1 is a SEMA-3A binding receptor and presents a very short cytoplasmatic tail with no signal-transducing motif, thus needing a co-receptor from the plexin family of transmembrane proteins with highly conserved intracellular domains [18].

SEMA-3A and NRP-1 are constitutively expressed by the thymocyte and thymic microenvironmental compartment, cooperating with thymocyte movement by inhibiting the chemoattractant migratory response induced by extracellular matrix elements – basically developing a chemorepulsive effect on thymocytes [9, 16].

Thymocyte migration within the thymic microenvironmental compartment is crucial for intrathymic T-cell development. Thymocyte migration is multivectorial and relies on the participation of chemokines, extracellular matrix elements, galectins, and semaphorins, among others [9, 10, 19, 20]. These molecules, conjointly, promote directional thymocyte migration through thymic cortex and medulla regions. In this context, our results are par-

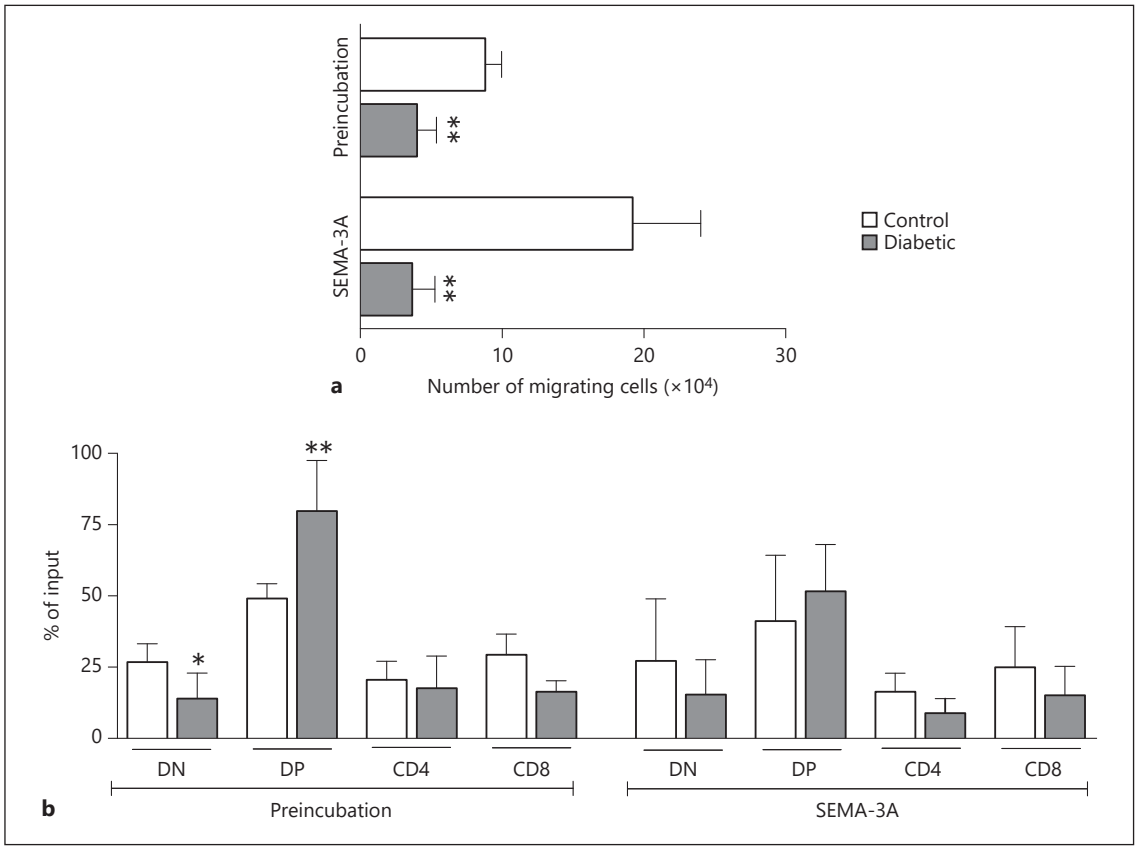

Fig. 5. Decrease in the semaphorin-3A (SEMA-3A)-driven chemorepulsive migratory response of thymocytes from chemically induced diabetic mice. **a** The total numbers of migrating cells under specific SEMA-3A stimulus (present during the assay, or pre-incubation plus washing before migration). **b** The same effect, seen in each CD4/CD8-defined thymocyte subset. The values shown correspond to specific migration after subtracting those numbers obtained for each individual in wells coated only with BSA, applied as an unrelated protein. Results are expressed as mean ± SE for at least 5 animals. * $p \leq 0.05$ and ** $p \leq 0.01$.

ticularly important because they clearly show alterations in the expression of SEMA-3A and NRP-1 in the thymus from chemically induced diabetic mice.

We previously showed that thymic atrophy under diabetic condition is followed by a massive drop of the DP thymocyte subset, loss of corticomedullary junction, and reduced expression of fibronectin and laminin receptors by thymocytes, as well as reduction in chemokine gene expression [14]. These results raised the hypothesis that chemorepulsive molecules might also be altered in the atrophic thymus from diabetic mice. Accordingly, we evaluated herein the effects of chemically induced diabetes on SEMA-3A/NRP-1 signaling/expression in thymocytes. No alterations in SEMA-3A expression by thymocytes between control and diabetic mice were observed. Thus, it is more plausible that the increase in SEMA-3A is predominantly driven by thymic epithelium. This is further supported by data showing that total thymus gene expression of SEMA-3A is high in the thymuses from diabetic mice, as is the case of NRP-1 and plexin-1 (data not show). It was previously demonstrated that SEMA-3A was increased in the suprabasal layer of the epidermis with high glucose level conditions, in both diabetic rats and humans, through the mTOR signaling pathway [21]. The mTOR pathway is evolutionarily conserved, and various cell types express mTOR [21]. Thus, it is not surprising that SEMA-3A is under mTOR signaling regulation in various types of cells [22–24]. In thymocyte development, mTOR is committed to integrating the immunological and environmental cues to shape T cell development [25]. Actually, all activities that drive T cells to integrate the immune response are driven by mTOR signaling activation to downstream effector pathways, which includes immune receptor signaling, metabolic programs, and migra-

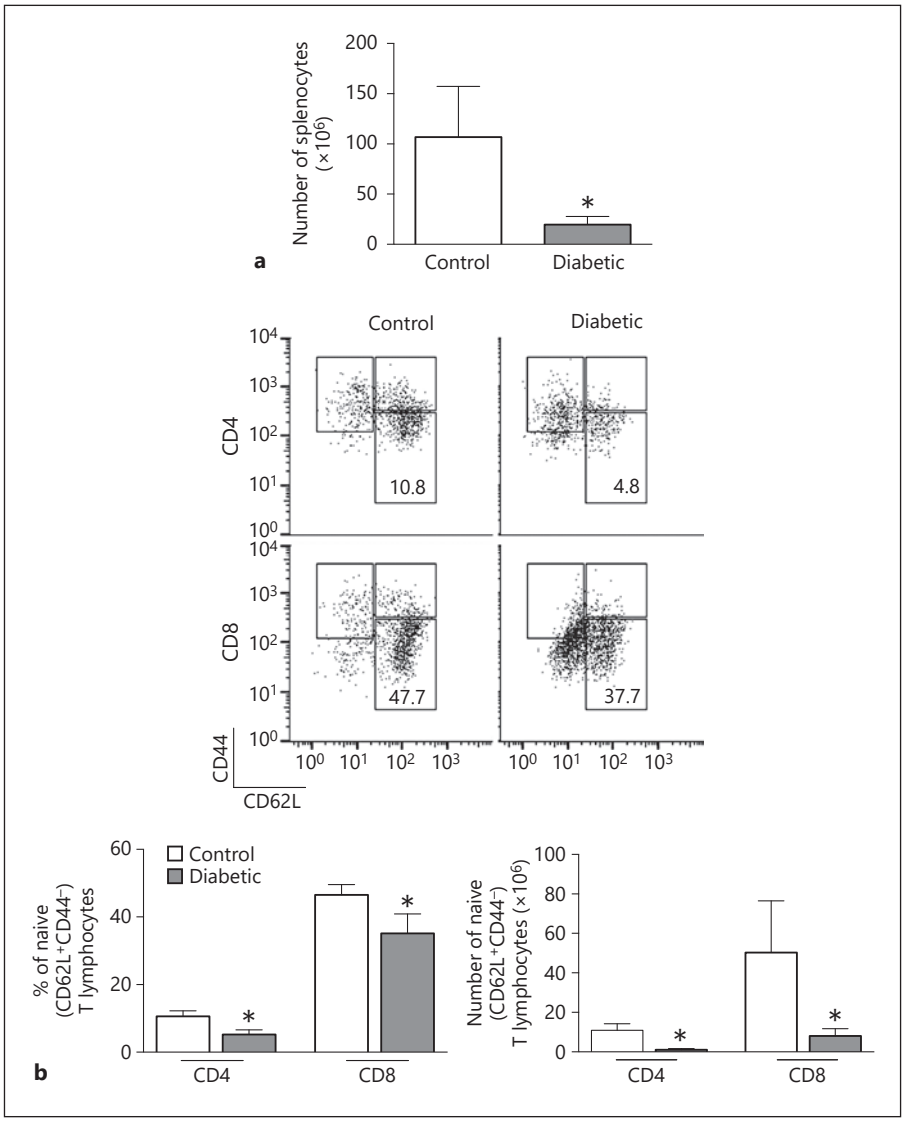

Fig. 6. Decrease in the numbers of splenic T lymphocytes in chemically induced diabetic mice. **a** The total numbers of splenocytes from control and diabetic groups. **b** The relative and absolute number of naïve $CD4^+CD62L^+CD44^-$ and $CD8^+$-$CD62L^+CD44^-$ T lymphocytes. Results are expressed as mean ± SE for at least 5 animals. * $p \leq 0.05$.

tory activity [26]. This evidence indicates that the SEMA-3A/NRP-1 alterations reported herein are related to the high glycemia seen in the diabetic mice. In a second vein, since Rutto et al. [27] demonstrated that in vitro SEMA-3A exhibits a negative effect upon TEC proliferation, it is plausible that the thymic microenvironment alterations that we previously observed are also secondary to high contents of SEMA-3A in the organ. The biological effects of SEMA-3A occurs upon binding this soluble molecule with the receptor complex comprised by 2 proteins: NRP-1 and plexin-A [28]. Under physiological conditions the engagement of SEMA-3A to the receptor complex NRP-1/plexin-A1 evokes the repulsion of thymocytes [10]. The repulsion is very important for intrathymic migration, especially for migration of the most conspicuous thymocyte subset, bearing the phenotype $CD4^+CD8^+$ [29]. Interestingly, here we found that DP was the thymocyte subpopulation showing the largest decrease in NRP-1.

Along with T-cell maturation, the adhesion and de-adhesion processes are essential for thymocyte migration and cell-cell interactions. Herein, we observed alterations with a decrease in SEMA-3A and NRP-1 expression patterns in thymocytes under diabetic condition, thus indicating changes in migration and prejudice for T-cell development. Actually, we observed a decrease in the chemorepulsive role of SEMA-3A, as revealed by transwell migration chambers. Such an effect was seen in all immature and mature thymocyte subsets from diabetic animals.

Taken together, our data clearly unravel a disruption in the normal cell migration pattern of developing thymocytes following chemically induced insulin-dependent diabetes, as ascertained by the altered migratory response to SEMA-3A. In conceptual terms, it is plausible to think that such disturbances in the migration pattern of thymocytes from these diabetic animals may exert an impact in the cell-mediated immune response of these mice.

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Statement of Ethics

All procedures were carried out in accordance with the guidelines proposed by the Brazilian Council on Animal Care (SBCAL) and approved by the animal care and use committee of the University of Campinas (protocol 2312-1).

Disclosure Statement

The authors declare that they have no conflicts of interest to disclose.

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Disclosure Statement

The authors declare that they have no conflicts of interest to disclose.

Author Contributions

Conception and design: C. Francelin, W. Savino, and L. Verinaud. Collection and assembly of data: C. Francelin and I. Geniseli. Data analysis and interpretation, writing of the manuscript, and final approval of the manuscript: all authors.

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