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Neuropilin 1 and CD25 co-regulation during early murine thymic differentiation

CATHERINE CORBEL^{†‡§¶}, VALERIE LEMARCHANDEL^{†‡§¶}, VERONIQUE THOMAS-VASLIN^{||‡},
ANNE-SOPHIE PELUS^{†‡§¶}, COLETTE AGBOTON^{†‡§¶} AND PAUL-HENRI ROMEO^{†‡§¶}

Correspondence and request for materials should be addressed to Catherine CORBEL
INSTITUT COCHIN, Département d'Hématologie
123, Boulevard de Port-Royal
75014 PARIS, France
Tel: 33-01-53 10 43 73, Fax: 33-01-43 25 11 67,
E.mail: corbel@cochin.inserm.fr

[†] Institut Cochin, Département d'Hématologie, Paris, F-75014 France

[‡] INSERM, U567, Paris, F-75014 France

[§] CNRS, UMR 8104, Paris, F-75014 France

[¶] Université Paris5, Faculté de Médecine René Descartes, UM 3, Paris, F-75014 France

^{||} CNRS, UMR7087, Pitié-Salpêtrière, CERVI, Paris, F-75013 France

[‡] Université Pierre et Marie Curie-Paris6, UMR7087, Pitié-Salpêtrière, Paris, F-75013 France

Abbreviations

BM: Bone marrow, **DC:** Dendritic cell, **DN:** CD4⁻CD8⁻ Double negative, **DP:** CD4⁺CD8⁺ Double positive, **NP:** Neuropilin, **Sema:** Semaphorin, **CD4-SP:** CD4⁺CD8⁻ Single positive, **CD8-SP:** CD4⁻CD8⁺ Single positive, **TN:** Triple negative, **Treg:** regulatory T cell, **TSC:** Thymic stromal cell, **VEGF:** Vascular endothelial growth factor.

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Abstract

Neuropilin 1 (NP1) is a receptor for both Semaphorin and Vascular Endothelial Growth Factor expressed by subpopulations of neuronal and endothelial cells. In the immune system, NP1 is present on dendritic and regulatory T cells. Here, we show that NP1 is expressed in the murine thymus, starting on day 12.5 of gestation. In the adult, NP1 is mainly expressed by CD4⁻CD8⁻ double negative cells, CD4⁺CD8⁺ double positive cells, and CD4⁺CD25⁺ regulatory T cells but barely detected in single CD4⁺ and CD8⁺ positive thymocytes. Within the CD4⁻CD8⁻CD3⁻ (TN) immature cells, NP1 expression starts in TN3 (CD44⁻CD25⁺) and increases in TN4 (CD44⁻CD25⁻) cells. In order to study the role of NP1 in thymocyte differentiation we generated mice in which the *np1* gene is selectively disrupted in the T cell lineage. The mutant mice display normal thymocyte, peripheral, conventional and CD4⁺CD25⁺Foxp3⁺ regulatory T-cell populations. However, we observe a down-regulation of the CD25 expression between the TN3 and TN4 stages that is (i) correlated to increased expression of NP1 in control mice and (ii) altered in mutant mice, suggesting that NP1 is co-regulated with CD25 expression during early immature thymocyte differentiation.

Keywords: rodents, T cell, cell surface molecule, thymus, dendritic cell, precursor, stroma.

1. Introduction

NP1 is a transmembrane glycoprotein, which plays an essential role in the establishment of both the nervous system and the endothelial network during embryogenesis [1,2]. NP1 is a receptor for two types of structurally unrelated ligands that mediates different functions. On neurons, NP1 was first shown to bind the class 3 secreted Semaphorin (Sema) 3A and allows the chemorepulsive guidance of developing axons [3]. On human endothelial cells, NP1 binds the 165 isoform of VEGF-A (VEGF165), enhances VEGF165 binding to VEGF receptor 2 (VEGFR2) and consequently induces both the migration and the proliferation of endothelial cells [4]. On both cell types, NP1 can bind the two ligands which exert opposite activities [5-8].

Neuropilin 2 (NP2) was cloned by homology with NP1 [9] and binds two VEGF isoforms, VEGF165 and VEGF145 and two Sema, Sema 3C and Sema 3F. During ontogeny of the nervous system NP2 shows different patterns of expression and induces the guidance of different axons [10,11]. In the developing vascular system, the two NP molecules are first expressed in blood islands. At later stages, NP1 is restricted to arterial endothelial cells while NP2 is expressed in venous and lymphatic endothelium [12-14].

Besides the neuronal and vascular systems, NP1 expression was also detected in the immune system. NP1 was actually shown to be present on human dendritic cells [15], murine hematopoietic fetal liver cells [16] and murine peripheral regulatory T (Treg) cells [17]. No data about NP1 expression and function during murine T-lymphoid differentiation have been yet reported.

During adult thymus development, immature $CD3^+CD4^+CD8^-$ (triple-negative, TN) precursor cells pass through four phenotypically distinct stages defined by expression of CD44 and the IL-2 receptor α chain (CD25) that is transiently expressed in DN thymocytes before differentiation in DP [18]: $CD44^+CD25^-$ (TN1), $CD44^+CD25^+$ (TN2), $CD44^-CD25^+$ (TN3) and $CD44^-CD25^-$ (TN4). After $\alpha\beta$ TCR rearrangement and positive and negative selection, $CD4^+CD8^+$ (DP) cells mature into $TCR\alpha\beta^+ CD4^+CD8^-$ or $TCR\alpha\beta^+ CD4^-CD8^+$ single-positive (SP) cells, the end product of the intrathymic $\alpha\beta$ T cell pathway [19,20].

In this study, we investigated the expression of NP1 during thymocyte differentiation and/or migration through the thymic microenvironment in adult and embryonic mice. In order to assess a role for NP1 in T lymphopoiesis we studied NP1 knock out mice. Since inactivation of the *np1* gene leads to embryonic lethality at around E10.5-12.5 [1,2], we developed a conditional knock out strategy in which *np1* gene was selectively deleted in the T cell lineage. This strategy was based on flanking the *np1* gene with Cre recombinase recognition sites (*loxP* sites) and expressing Cre under the control of a T cell-specific $p56^{lck}$ promoter.

We showed that in normal mice NP1 expression increased during the progression of thymocyte differentiation from the TN3 to the TN4 stage in association with the down-regulation of CD25 expression, which was altered in the conditional NP1 knock out mice. However, thymic cells populations and peripheral T lymphocytes were not significantly affected in these mutant mice.

2. Materials and methods

2.1. Mice

Eight-week-old and pregnant C57BL/6J mice were purchased from Harlan (France). Mice carrying loxP sites in the np1 gene (np1-flox) were provided by Dr. Ginty's laboratory [21] and the Lck-Cre Tg mice, which express the Cre coding sequence under the control of the p56^{lck} promoter, were obtained from Dr. Rabbitts's laboratory [22]. Mutant mice expressing the Lck-Cre transgene and homozygous at the floxed np1 locus (Tg np1^{-/-} mice) were generated by mating np1-floxed mice with Lck-Cre Tg mice. Their genotype was determined by PCR assays on tail DNA using previously described primers and amplification conditions [21,23]. The littermates used as controls with a normal NP1 expression were Lck-Cre Tg with a heterozygous np1-floxed locus (Tg np1^{+/-}), Lck-Cre Tg with a wild type np1 locus (Tg np1^{+/+}) or non Tg with a homozygous np1-floxed locus (non-Tg np1^{-/-}) mice.

2.2. Immunohistology on sections

Embryos were fixed in 4% (w/v) paraformaldehyde, embedded in gelatin-sucrose and frozen in isopentane at -80 °C. These were then sliced into 10 µm sections. Antibody staining and immunoperoxidase tissue analysis on the cryostat sections were carried out as previously described [24], except that a biotinylated secondary Ab and streptavidin coupled to horseradish peroxidase were used.

2.3. Preparation of cell suspensions

Thymus and spleen cell suspensions were prepared by teasing the organs on a 200-mesh stainless steel screen. PBL were obtained by retro-orbital bleeding under anesthesia and red blood cells were depleted by Ficoll gradient. The cells were washed and resuspended at 4°C in PBS containing 3% FCS.

DCs were generated from bone marrow (BM) according to the procedure previously described [25], with some modifications. Briefly, BM cells from femurs and tibias were flushed with a 25-gauge needle and placed in Petri tissue culture dishes for 2h at 37°C in complete medium (CM). CM consists of RPMI 1640 medium supplemented with 10% of heat-inactivated FCS, 10 mM HEPES, 2mM glutamine, 1% kanamycin (Gibco, Invitrogen Corporation) and 50 μ M 2-ME (Sigma-Aldrich, St. Louis, MO). Nonadherent cells were collected and transferred into culture flasks containing CM supplemented with recombinant murine GM-CSF (10ng/ml) and IL-4 (1ng/ml) (R&D Systems, Minneapolis, MN). Two-thirds of the medium was replaced on day 3. On day 5 or 6, CD11c⁺ cells were purified using anti-CD11c-coated immunomagnetic beads (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions. DC maturation was induced by adding 1 μ g/ml of Escherichia coli-LPS (Sigma, St. Louis, MO), for 24 or 48 additional hours.

Thymic stromal cells (TSC) were obtained as essentially described [26]. Thymic tissue was enzymatically digested and cultured for 3 to 4 wks in CM. Absence of CD45 expressing cells in the culture was checked by immunolabelling and FACS analysis.

2.4. Antibodies, flow cytometric analysis and cell sorting

FITC-, PE-, PerCP- or PE-Cy5-labeled mAbs RM4-5 (anti-CD4), 53-6.7 (anti-CD8a), 17A2 (anti-CD3), PC61 (anti-CD25), 30F11 (anti-CD45), 1D3 (anti-CD19), M1/70 (anti-CD11b), PK136 (anti-NK1.1), IM7 (anti-CD44), 7D4 (anti-CD25), HL3 (anti-CD11c). Unlabeled 2.4G2 (anti-CD16/CD32) and labeled isotype control mAbs were all purchased from PharMingen (San-Diego, CA). Three goat polyclonal anti-rat neuropilin Abs were used: two anti-NP1 Abs, GT15036 (Neuromics Antibodies, Northfield, MN) and AF 566 (R&D Systems, Minneapolis, MN) and one anti-NP2 Ab, AF 567 (R&D Systems, Minneapolis, MN). All were revealed using a PE-labeled donkey anti-goat Ab (Jackson ImmunoResearch, West Grove, PA). A rabbit anti-mouse NP1 Ab produced and provided by Dr. H. Fujisawa [27] was also used with a PE-labeled donkey anti-rabbit Ab (Jackson ImmunoResearch, West Grove, PA).

Surface Fc receptors were preliminary blocked by pre-incubation with anti-CD16/CD32 mAb for 10 min. Cells were incubated for 30 min at room temperature with mAbs and washed in FCS containing medium. After staining with polyclonal Abs, cells were incubated another 30 min with the labeled secondary Ab and washed twice. Foxp3 intracellular staining with the anti-mouse/rat Foxp3 staining set (clone FJK-16s) from eBioscience (Clinisciences, France) was done according to the manufacturer's guidelines, after cell surface staining.

Triple negative (TN) thymocytes were isolated according to a two-step method. Thymic cells reacting to anti-CD4 and anti-CD8 were removed by depletion with

immunomagnetic beads (CD4 and CD8a microbeads, MACS, Miltenyi Biotec, France) following the manufacturer's guidelines. Cells were subsequently stained with a cocktail of conjugated mAbs i.e., anti-CD3, -CD4, -CD8, -CD19, -CD11b and -NK1.1 and all negative cells were sorted. Such purified TN thymocytes were further surface triple-stained with a polyclonal Ab against NP1, then with a secondary Ab conjugated to PE and finally with a mAb APC-anti-CD44 together with a mAb FITC-anti-CD25. Analyses were performed using a FACScan flow cytometer and CellQuest software (Becton Dickinson) and sorting was performed using a Epics Altra instrument (Coulter). Foxp3/CD25/CD4/CD8 analysis was performed on a FACSibur (Becton Dickinson), using Flowjo software (Stanford University, Tree Star, Inc.).

2.5. Western blot analysis

Total protein extracts were obtained in a lysis buffer containing 137mM NaCl, 20mM Tris HCl at pH 8.0, 2.7 mM KCl, 10% glycerol and 1% NP40. Briefly, cells were collected, washed in cold PBS, then resuspended in cold lysis buffer supplemented with anti-proteases (Boehringer Mannheim), incubated in ice for 15 min and centrifuged at 20,000g for 15 min. Supernatants were used directly for immunoblotting. Proteins (25 mg in each lane) were separated by SDS-PAGE (12%) and were transferred onto a Hybond-P PDVF membrane (Amersham, Piscataway, NJ) by electroblotting. Immunoreactive proteins were detected with a goat anti-VEGF Ab AF 564 (R&D Systems, Minneapolis, MN) at a dilution of 1:1,000. The blot was revealed by a chemiluminescence detection system (Amersham) according to the manufacturer's protocol.

2.6. Analysis of data

All experiments were performed using 3 to 10 mice and data shown are means $\pm$ SD. Statistical analysis was conducted using Student's unpaired t-test. P values higher than 0.05 differences between test groups were deemed not significant.

3. Results

3.1. NP1 expression by murine dendritic cells and thymocytes

We investigated whether NP1 and NP2 were expressed in the murine adult immune system. Flow cytometric analysis was first performed on murine CD11c⁺ DC generated *in vitro* from BM precursor cells isolated from normal C57BL/6 mice. Such immature DC (iDC) weakly expressed NP1 and NP2 (0.5 to 5%). However, when these cells were incubated with LPS to trigger DC maturation, 45% of mature DC (mDC) expressed NP1 (Fig. 1A). We also showed that mDC expressed NP2 and that NP2⁺CD11c⁺ cells represented 79% of mDC (Fig. 1A). Neither NP1 nor NP2 expression could be detected in resting T lymphocytes from peripheral organs such as spleen, lymph nodes and blood but, in the spleen, 43% of CD4⁺CD25⁺ Treg cells expressed NP1 (data not shown).

Then we investigated whether neuropilins were expressed in the C57BL/6 thymus. We showed that NP1 but not NP2 was expressed by adult thymocytes (Fig. 1B). Differences in the percentage of NP1 expressing cells were observed that depended on the Ab used as illustrated in Figure 1B. The mean value (from 6 mice) of NP1 positive cells was 41% $\pm$ 8% when Abs against rat NP1 (AF 566 and GT15036) were used whereas it was 63% $\pm$ 10 % (from 8 mice) using the species-specific anti-mouse Ab [27].

From these results, we conclude that murine mDC express both NP1 and NP2 molecules whereas adult thymocytes only express NP1.

3.2. NP1 and VEGF expression in thymic cell populations

We thereafter focused our study on thymic differentiation. NP1 expression in thymocytes was assessed by immunohistochemistry on thymus sections. Specific NP1 staining was localized primarily to the cortex and the subcapsular zone (SCZ) of the thymus. In the medulla, where mature SP thymocytes reside before export, NP1 staining was less apparent (Fig. 2A, C). This pattern of expression suggested that DP and DN thymocytes expressed NP1. The staining of consecutive sections with ER-TR4 (Figure 2B and 2D), a mAb specific for cortical thymic epithelial cells (EPC) [28], reveals that cortical EPC did not express NP1. Medullary EPC and thymic mesenchymal cells were also NP1 negative (data not shown). Thus, NP1 expression is specific of hematopoietic cells in the thymus.

Since the VEGF₁₆₄ isoform of VEGF-A is a ligand for NP1 on murine endothelial cells [4] and is also involved in hematopoietic and immune regulation [29], we investigated its expression in the thymus by western blot assays. We analysed two thymic cell populations: thymocytes and cultured non-hematopoietic thymic stromal cells (TSC). Since production of VEGF-A was previously observed in activated human DC [30,31] we also investigated VEGF-A expression by DC, immature (iDC) and mature (mDC), obtained from cultured BM cells as described herein, as a positive control. No expression could be detected in iDC whereas upon LPS activation, mDC expressed both VEGF₁₂₀ and VEGF₁₆₄ isoforms (Fig. 2E). Interestingly, we found that these two VEGF isoforms were produced by TSC but not by thymocytes (Fig. 2E).

These results demonstrate that the VEGF₁₆₄, a potent ligand for NP1, is expressed by TSC but not by thymocytes.

3.3. NP1 expression during thymocyte differentiation

In the adult, NP1 expression was studied in the four thymic different subpopulations defined by the expression of the CD4 and CD8 markers. Among NP1⁺ thymocytes, 90% were DP and 6% were DN cells (NP1⁺ cells are represented by black dots and NP1⁻ cells by grey dots in Figure 3). Analysis of NP1 expression in gated subpopulations revealed that half of DN (54%) and DP (42%), but only 2 to 3% of CD4 and CD8 SP cells expressed NP1. Furthermore, two populations of NP1⁺ cells with low and high fluorescence intensity could be observed particularly in DN cells. We also found NP1 expression in 62% of thymic CD4⁺CD8⁻CD25⁺ Treg cells (data not shown).

Since during early ontogeny, i. e., from 12.5 days of gestation (E12.5) to E14 all thymocytes are DN cells [32], NP1 expression was also studied at those stages. Within CD45⁺ hematopoietic cells, NP1 expression started at E12.5 (2%), and then increased. At E13.5 and E14, 34% and 61% of CD45⁺NP1⁺ cells were detected, respectively. Furthermore, the intensity of NP1 expression increased at E14 and CD45⁻ cells (TSC) did not express NP1 (Fig. 4).

Altogether, these results show that NP1 expression starts at E12.5 during embryonic development in thymic hematopoietic cells and is maintained during thymocyte differentiation in adult life. NP1 is expressed by around half of thymocytes especially by DN, DP and CD4⁺CD25⁺ Treg cells.

3.4. T cell subpopulations in the absence of NP1

In order to study the role of NP1 in thymic differentiation we generated conditional knock out mice whose *np1* gene was selectively disrupted in T cells using a Cre-loxP strategy. Mice in which exon 2 of the *np1* gene was flanked by *loxP* sites [21] were crossed with transgenic mice expressing the Cre recombinase under the control of the *lck* promoter which is active in T cells from DN1 stage to all subsequent T cell differentiation stages [22].

Homozygous mutant knock out mice, (Tg *np1*^{-/-}), were viable, fertile, normal in appearance and size in comparison to the controls. Their thymic size and cellularity were also similar to the control littermates. We first checked by FACS analysis that the Tg *np1*^{-/-} thymocytes had efficiently excised the floxed allele. In 10 Tg *np1*^{-/-} mice, NP1 staining was actually found in only $2.5\% \pm 1.3\%$ thymocytes as compared to $50\% \pm 10\%$ in 8 littermate controls (data not shown).

As shown in Table 1, Tg *np1*^{-/-} mice displayed normal percentages of thymocytes and consequently normal percentages of peripheral CD8 and CD4 T lymphocytes. In the thymus, the percentage of $\alpha\beta$ TCR positive cells of Tg *np1*^{-/-} mice was also identical to that of control mice (data not shown).

Since among mature T cells, NP1 expression was restricted to thymic and peripheral CD4⁺CD25⁺ Treg cells, we asked whether the absence of NP1 may alter the development of this cell subset. The transcriptional factor Foxp3 being a marker of the Treg cell population, Foxp3 expression was analysed by intranuclear staining in flow cytometry together with CD25 expression. Similar percentages of Foxp3⁺CD4⁺CD25⁺ cells from thymus and spleen were observed in

Tg *np1*^{-/-} and control mice (Fig. 5). Foxp3 was not detected, by FACS, in thymic DN/TN cells (Fig. 5).

3.5. NP1 and CD25 co-regulation during TN thymocyte differentiation

Careful examination of CD25 expression showed that in immature CD4⁺CD8⁻ thymocytes, invalidation of *np1* altered the level of CD25 expression (Fig. 5, upper panel). Thus, in DN thymic cells and in absence of NP1 expression, CD25^{hi} cells accumulated, while the proportion of CD25^{lo} and CD25⁻ cells were reduced. This result suggests that NP1 expression may be required for CD25 down-regulation in DN cells. Thus we further investigated the expression of NP1 in the most immature thymocytes according to CD25 and CD44 expression. Knowing that DN cells contain CD3⁺ mature cells, TCR $\gamma\delta$ ⁺ and TCR $\alpha\beta$ ⁺NK1.1⁺ (NKT) cells [33,34], we sorted CD4⁺CD8⁻CD3⁻ cells, termed triple negative (TN) [35]. We analysed the four TN subsets of Tg *np1*^{-/-} and control littermate mice. In *np1*^{-/-} mice, although we confirmed that the CD25 down-modulation is altered in TN cells, the percentages of TN1, TN2, TN3 and TN4 cells were not significantly different ($P > 0.05$, Student's unpaired *t*-test) whatever NP1 was expressed or not (Fig. 6A). We examined NP1 expression and CD25 in the TN subpopulations from control littermate mice. NP1 was mainly expressed in CD25⁻ TN cells (Fig. 6B). NP1⁺ cells were mainly present at the TN3 stage (14% NP1⁺ cells) and at the TN4 stage (72% NP1⁺ including 11% NP1^{hi} cells). Interestingly, the increase of NP1 expression during maturation from TN3 to TN4 stages was correlated to the decrease of CD25 expression (Fig. 6B).

Altogether, these data demonstrate that the increase of NP1 expression during maturation of thymocytes from TN3 to TN4 stages is related to CD25 down-regulation and that absence of NP1 expression alters such a process.

4. Discussion

Searching for the expression of neuropilins in the murine immune system, we identified the thymus as a novel organ, which contains hematopoietic NP1 expressing cells.

In the adult thymus, NP1 expression is found on CD45⁺ hematopoietic cells but not on epithelial nor mesenchymal thymic stromal cells, and is localized mainly in the cortex and the SCZ. DN progenitors from BM are thought to enter into the thymus at the cortico-medullary junction and subsequently to migrate to the SCZ during DN development. The migration from cortico-medullary junction to SCZ depends on differentiation-inducing signals in defined cortical regions [36]. We have shown that TN3 and TN4 cells express NP1. These cells can be found in the cortex and the SCZ [36,37], sites that appear to be positive for NP1 staining. Moreover, in accordance with its cortical localization, NP1 expression was found in around half of DN and DP thymocytes.

Interestingly, a high frequency of NP1 expression was found on large thymocytes and at stages where intense proliferative activity was previously reported [38,39]. NP1 expression is actually almost undetectable in CD44⁻CD25^{hi} cells (early resting TN3 stage), it increases (14% NP1⁺) in CD44⁻CD25^{lo} cells (late cycling TN3 stage) and it peaks (72% NP1⁺) in CD44⁻CD25⁻ cells (highly cycling late TN4 stage). Thereafter, at the DP stage, NP1 expression decreases in correlation with a lower frequency of cycling cells at this stage. Finally, NP1 is almost undetectable in SP mature cells that are mainly resting cells.

Initiation of NP1 expression occurs at the TN3 stage, when TCR β genes rearrange and pair with the preTCR α chain (pT α) to form the preTCR complex. Signalling through the CD3 components of the preTCR complex delivers a signal that down-regulates the CD25 expression on TN3 cells and promotes their development, via TN4, to the DP stage. Therefore, up-regulation of NP1 expression i.e., increase of NP1⁺ cell frequency and intensity of cell surface expression, occurs during DN3 to DN4 differentiation in relation with the loss of CD25. It was previously shown that CD25 down-regulation is dependent on the expression of the TCR and is compromised in several models of immunodeficient mice [40-42]. Here, we show that decrease of CD25 expression is also linked to NP1 expression in normal thymocytes and is altered in the absence of NP1 in Tg np1^{-/-} mice. NP1 expression is maximal at DN4 stage and down-regulation starts on DP stage.

Confirming the NP1 expression in immature adult thymocytes, we observed increases in the proportion of NP1 expressing cells and of the intensity of NP1 expression from 12.5 to 14 days of embryonic development, in the hematopoietic component of the thymus. At these embryonic stages all thymocytes still belong to the DN/TN population but display heterogeneous CD44 and CD25 expression. In the C57BL/6 mouse strain studied, at E12, thymocytes are all DN1 [43]. At E14, DN1 to DN4 stages are observed [44]. At E15, CD8⁺CD4⁻CD3⁻ cells emerge while TCR⁻CD8⁻CD4⁺ cells remain almost undetectable throughout gestation [32]. The frequency of NP1 positive cells drops in mature SP thymocytes in accordance with the lack of NP1 expression by conventional peripheral T cells shown herein

and previously described [17]. The few CD4-SP thymocytes expressing NP1 (2.5%) may correspond, in part, to the Treg cell population that is shown herein to express NP1 and/or to the CD4⁺ type I NKT cells [45]. A low frequency of NP1 cells is also observed in CD4⁻CD8⁺ thymic cells. These cells may correspond to an immature single positive stage before differentiation in DP stage [46,47]. Moreover we show that murine DC obtained from BM express both NP1 and NP2 and that this expression requires cell activation in the presence of LPS. Recently, NP1 expression was also found in murine DC exosomes, which are secreted vesicles formed in late DC endocytic compartments [48]. Since thymus also contains DC stained with CD8 α [49], we cannot exclude that NP1⁺CD8⁺ thymic cells could also contain DC expressing NP1.

The differentiation/proliferation/migration phases of thymocytes are dependent on interactions with different stromal cell types. NP1 on the surface of thymocytes may act in regulating thymopoiesis during the cross-talk with stromal compartments. We show on sections that TSC, which comprise epithelial and mesenchymal cells, do not express NP1. Since VEGF-A, a ligand for NP1, first described as an endothelial cell-specific factor, also affects neurons, neural progenitors and astrocytes (for review see [50], we investigated its expression by thymic cell populations. We show that TSC but not thymocytes express VEGF-A (VEGF120 and VEGF164). These results are in agreement with the recently described expression of *vegf* mRNAs encoding VEGF120, VEGF164 and VEGF188 by both thymic epithelium and mesenchyme [51]. Our results suggest that VEGF164 produced by TSC may bind NP1 on thymocytes. We hypothesise

that, in the thymus, VEGF-A may promote interactions between stromal elements and NP1 expressing thymocytes, essentially late DN/TN and early DP cells.

We extend to C57BL/6 thymic and splenic Treg cells the initial observation about NP1 expression by splenic Treg cells of Balb/c mice [17]. Treg cells are mediators of dominant immune tolerance to self [52] and some of us have previously shown the role of thymic epithelium in positive selection of CD4 regulatory T cell [53,54]. Murine CD4⁺CD25⁺ Treg cells development as an independant subset of CD4⁺ thymocytes was further confirmed [55]. Foxp3, a transcriptional factor [56] is required for their specific function of suppression [57].

In mice deficient for NP1 in the T cell lineage, Treg development was not affected in either thymus or periphery since Foxp3⁺CD4⁺CD25⁺ Treg cell numbers were similar to that in controls. Bruder et al.[17] suggested that NP1 gene expression is regulated by the transcription factor Foxp3. Here we show that such a regulation cannot occur in immature DN/TN cells since Foxp3 protein expression is undetectable in these cells. Moreover, analysis of our NP1 knock out mutant mice suggests that NP1 has no mandatory role in Treg function because even elderly mice do not exhibit any autoimmune disease. Although the absence of NP1 expression in the T cell lineage results neither in difference in the number of total thymocytes, nor peripheral T cells numbers, it leads to a discrete alteration of thymocyte differentiation upstream at the TN3 stage. In the absence of NP1 expression there is a partial block in the down-regulation of CD25 expression between TN3 and TN4 stage. This observation is in agreement with synchronized

down-regulation of CD25 expression and up-regulation of NP1 expression in normal C57BL/6 mice.

Altogether, we showed the regulated transient NP1 expression in thymus during DN/TN and DP differentiation, and its synchronisation with CD25 down-regulation, but the absence of NP1 during early thymocyte differentiation does not compromise further T cell differentiation.

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Figure legends

Figure 1. Cell surface expression of neuropilins on DC and adult thymocytes.

NP1 and NP2 flow cytometric analyses of mature DC (mDC) obtained from BM cell culture and following maturation with LPS (A) and thymic (B) cellular suspensions. Filled histograms represent staining with polyclonal Abs directed against rat NP1 (AF 566), rat NP2 (AF 567) or mouse NP1(*) [27]. Open histograms are the same cell populations stained with control Ig: goat Ig and PE-donkey anti-goat Ig for NP1 and NP2 Abs, rabbit Ig and PE-donkey anti-rabbit for NP1* Ab. Results are from one representative experiment out of six to eight.

Figure 2. Expression of NP1 and VEGF in the thymus.

A-D. Immunohistochemical analysis of NP1 expression.

A, B. Consecutive thymic sections. C, D. Higher magnification of A and B.

A, C. NP1 expression was detected using AF 566 Ab.

B, D. Medullary epithelial cells were detected using ER-TR4 mAb. M, medulla;

C, cortex; SCZ, subcapsular zone.

E. Immunoblot analysis of VEGF-A expression.

In thymus: TSC and thymocytes. In BM-derived DCs: iDC and mDC.

VEGF-A isoforms are VEGF 164 (21kD) and VEGF 120 (14 kD).

Figure 3. Cell surface expression of NP1 on thymocyte subsets from adult mouse.

Flow cytometry analysis of adult thymic cells stained with anti-CD8, anti-CD4 mAbs and anti-NP1 Ab. Upper panel showed the different thymic populations characterized by CD4 and CD8 expression. Numbers in quadrants represent percentages of cells. Black and grey dots represent NP1⁺ and NP1⁻ gated cells, respectively. Lower panel showed NP1 expression in the different thymic populations. NP1 expression for each gated subset: DN, DP, CD4-SP and CD8-SP is represented by filled histograms. The numbers in histograms indicate the percentage of NP1^{lo} and NP1^{hi} fluorescent cells. Overlay open histograms are the same cell populations stained with control Ig. Results are from one representative experiment.

Figure 4. Surface NP1 expression by embryonic thymic cells.

Flow cytometry analysis of E12.5 to E14 thymus double stained with anti-CD45 and anti-NP1. Numbers represent percentages of NP1 positive cells.

Figure 5. CD25/Foxp3 expressing cells in Tg np1^{-/-} mice.

FACS analysis of thymic and splenic cells from control C57BL/6 and Tg np1^{-/-} mice using CD4, CD8, CD25 cell surface staining and intranuclear Foxp3 staining. Expression of CD25 and Foxp3 is shown in CD4⁻CD8⁻ thymic cells and in CD4⁺CD8⁻ cells from thymus and spleen. Numbers indicate percentages of cells.

Figure 6. Triple negative (TN) thymic subpopulations in Tg np1^{-/-} and control mice.

Thymocytes negative for expression of CD4, CD8, CD3, CD11b, CD19 and NK1.1 were purified by cell sorting and termed TN cells.

A. TN cells were stained with anti-CD44 and anti-CD25 mAbs and the percentages of CD44⁺CD25⁻ (TN1), CD44⁺CD25⁺ (TN2), CD44⁻CD25⁺ (TN3) and CD44⁻CD25⁻ (TN4) cells were determined. The means $\pm$ SD are plotted for Tg np1^{-/-} (n=3) and littermate controls (n=3). (P>0.05).

B. TN subpopulations from control littermates were surface-stained with anti-CD44, CD25 and NP1 Abs. NP1⁺ gated cells were shown as black dots and NP1⁻ cells as grey dots in CD25/CD44 dot plot.

NP1 expression in TN3 and TN4 gated subsets is represented by filled histograms and isotypic control by overlay open histograms. Numbers indicate percentages of NP1^{lo} and NP1^{hi} cells. The mean fluorescence intensity of NP1⁺ cells is 36 and 52 in TN3 and TN4 cells, respectively.

Table 1. T cell subpopulations in Tg np1^{-/-} mice

Mice	Percentage of thymic and peripheral T cells (% $\pm$ SD)					
	Thymus				Spleen	
	DP	CD4-SP	CD8-SP	DN	CD8-SP	CD4-SP
Control* (n=8)	83.9 $\pm$ 4.8	10.7 $\pm$ 3.1	1.4 $\pm$ 0.9	3.8 $\pm$ 1.6	12.0 $\pm$ 2.5	17.2 $\pm$ 6.1
Tg np1 ^{-/-} (n=10)	81.4 $\pm$ 8.6	12.6 $\pm$ 6.6	0.9 $\pm$ 0.5	4.6 $\pm$ 1.9	11.4 $\pm$ 4.1	17.9 $\pm$ 4.4

* Littermate mice were used as controls (see materials and methods).

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6