

# Neuropilin-1 expression identifies a subset of regulatory T cells in human lymph nodes that is modulated by preoperative chemoradiation therapy in cervical cancer

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## Summary

We examined the phenotype and function of CD4<sup>+</sup> T cells expressing the semaphorin III receptor neuropilin-1 (Nrp1) in human lymph nodes and peripheral blood. In lymph nodes, Nrp1 identified a small regulatory CD4<sup>+</sup> CD25<sup>high</sup> T-cell subpopulation (Nrp1<sup>+</sup> Treg) that expressed higher levels of Forkhead box P3 (Foxp3) message and protein than Nrp1<sup>-</sup> Treg, and various molecular markers of activated Treg, i.e. CD45RO, human leucocyte antigen (HLA)-DR and glucocorticoid-induced tumour necrosis factor receptor (GITR). Similarly to conventional Treg, Nrp1<sup>+</sup> Treg proliferated poorly *in vitro*, and exerted contact-dependent *in vitro* suppression of T-cell proliferation and cytokine secretion. However, Nrp1<sup>+</sup> Treg were more efficient than Nrp1<sup>-</sup> Treg at inducing suppression. Nrp1 was also expressed on a small subpopulation of CD25<sup>int</sup> and CD25<sup>-</sup> CD4<sup>+</sup> T cells that expressed more Foxp3, CD45RO, HLA-DR and GITR than their Nrp1<sup>-</sup> counterparts. In contrast, in peripheral blood Nrp1 identified a minor CD4<sup>+</sup> T-cell subset that did not display the phenotypic features of Treg lacking Foxp3 expression and marginally expressing CD25. Hence, the function of Nrp1<sup>+</sup> CD4<sup>+</sup> T cells seemingly depends on their anatomical location. In a previous report, we proposed that Treg may curb the anti-tumour T-cell response in cervical cancer. We show here that Treg and Nrp1<sup>+</sup> Treg levels dropped in the tumour-draining lymph nodes of patients with cervical cancer following preoperative chemoradiotherapy in a direct relationship with the reduction of tumour mass, suggesting that suppressor cell elimination facilitated the generation of T cells mediating the destruction of the neoplastic cells left behind after cytotoxic therapy.

**Keywords:** cervical cancer; chemoradiation; human lymph node; neuropilin-1; regulatory T cell

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## Introduction

CD4<sup>+</sup> T cells that constitutively express the interleukin-2 (IL-2) receptor  $\alpha$ -chain CD25 (regulatory T cells, Treg) and the master regulator Foxp3 transcription factor play a central and prominent role in maintaining self-tolerance

and in regulating responses to infectious agents, transplantation antigens and tumour antigens.<sup>1–3</sup> In humans, Treg are mostly present in the ~ 2–3% of CD4<sup>+</sup> cells that express the highest levels of CD25,<sup>4</sup> yet even the CD4<sup>+</sup> CD25<sup>high</sup> regulatory population is heterogeneous, both phenotypically and functionally. For example,

Abbreviations: APC, allophycocyanin; CC, cervical cancer; cDNA, complementary DNA; CFSE, carboxyfluorescein diacetate succinimidyl ester; CR, chemioradiotherapy; DEPC, diethylpyrocarbonate; ECD, PE-Texas red; ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein isothiocyanate; Foxp3, forkhead box P3; GITR, glucocorticoid-induced tumour necrosis factor receptor; HLA, human leucocyte antigen; IFN- $\gamma$ , interferon- $\gamma$ ; IL-2, interleukin-2; LN, lymph nodes; mAbs, monoclonal antibodies; mRNA, messenger RNA; Nrp1, neuropilin-1; NT, not treated; PB, peripheral blood; PBL, peripheral blood lymphocytes; PC5, PE-cyanin 5-1; pDC, plasmacytoid dendritic cells; PE, phycoerythrin; PHA, phytohaemagglutinin; PR, pathological response; RT-PCR, real-time reverse transcription-polymerase chain reaction; TDLN, tumour-draining lymph nodes; Treg, regulatory T cells.

20–30% of these cells express class II major histocompatibility complex and represent a distinct, particularly powerful Treg subset.<sup>5</sup> A variety of additional signature proteins, including CD62L, CD45RO, CD103, glucocorticoid-induced tumour necrosis factor receptor (GITR), and a variety of chemokine receptors are expressed by Treg,<sup>6,7</sup> but they have not been associated with any specific Treg subset. Moreover, there is increasing evidence that regulatory cells can be found also among CD4<sup>+</sup> CD25<sup>int/neg</sup> cells.<sup>8,9</sup>

Neuropilin-1 (Nrp1), originally described as a cell surface glycoprotein that acts as a semaphorin III receptor that is fundamental to neurological synapses,<sup>10,11</sup> plays a crucial role in the regulation of immune responses.<sup>12</sup> Earlier evidence from human studies<sup>13</sup> indicated that Nrp1 participates at the immunological synapse, promoting dendritic cell–CD4<sup>+</sup> T-cell clustering via homotypic interaction and leads to immune activation. In contrast, a later report in mice showed that Nrp1 is an exclusive feature of Treg,<sup>14,15</sup> and is therefore associated with suppressive rather than effector activity. Although this discrepancy may question to what extent studies in mice can be extrapolated to humans and should raise a cautionary note about the actual significance of Nrp1 expression in human T cells, Nrp1 is usually referred to as a marker of human Treg.<sup>16,17</sup> Moreover, studies in mice were performed in lymphoid organs and not in peripheral blood (PB) as in humans.

Thus, we sought to identify human CD4<sup>+</sup> T cells expressing Nrp1 in lymph nodes (LN) and PB, and to verify whether Nrp1 expression depended on the anatomical site and predicted cell function. Here we demonstrate that in human LN Nrp1 expression distinguishes a powerful suppressive Treg subset that is anergic and inhibits T-cell proliferation and cytokine production via a contact-dependent mechanism that is associated with Foxp3, whereas Nrp1<sup>+</sup> CD4<sup>+</sup> T cells in human PB are exceedingly rare, and do not express the Treg markers CD25 and Foxp3.

Strong evidence is emerging that Treg thwart the immunogenicity of neoplastic cells counteracting the development and effector functions of cytotoxic T cells.<sup>3</sup> Thus, in the second part of the investigation, we studied whether Nrp1<sup>+</sup> Treg could be relevant in the context of the immune response occurring in tumour-draining LN (TDLN). Cervical cancer (CC) is a gynaecological tumour under investigation as a possible candidate for immunotherapeutic strategies.<sup>18,19</sup> The clinical management of CC consists of radical surgery, or radiotherapy in early-stage tumours, and exclusive concomitant chemoradiotherapy (CR) in locally advanced tumours.<sup>20</sup> Investigational multi modal strategies incorporating radical surgery as adjuvant treatment following CR have also been explored.<sup>21,22</sup> This approach makes CC a suitable clinical context to study the immune status of TDLN and the modulatory effects of therapeutic intervention. In a previous report,<sup>23</sup> we

proposed that the decreased Treg frequency and the concomitant recruitment/expansion of effector T and natural killer cells seen in TDLN from preoperative CR-treated CC patients contributed to the primary tumour mass reduction. Here, we have extended that study by showing that CR depletes TDLN of Nrp1<sup>+</sup> Treg in direct relationship with a favourable response to the treatment.

## Materials and methods

### *Tissue sample and cell isolation*

The study was approved by the ethical committee of the Catholic University. Written informed consent was obtained from all patients. Because the study of human LN from healthy individuals is limited by ethical considerations, four LN taken from patients (aged 32–65 years) with benign diseases undergoing lymphadenectomy as a diagnostic procedure were used. In addition, four upper pelvic LN from patients with early-stage endometrial cancer were used for the study because they were distant from the location of the primary tumour. The lymphocyte composition of these LN has been found to be close to normal in a previous study.<sup>24</sup> All cancer patients underwent lymphadenectomy as part of their primary surgical treatment, were free of nodal and haematogenous metastases and did not receive any medications before surgery. This study also included a total of 14 CC patients admitted to the Gynecologic Oncology Unit. Cases with early-stage disease (FIGO Stage IB–IIA, major tumour diameter < 4 cm) were primarily submitted to radical surgery [not treated (NT) patients,  $n = 5$ ], whereas locally advanced cases ( $n = 9$ ) were administered preoperative platinum-based CR, as described elsewhere.<sup>23</sup> Four to five weeks after the end of treatment, patients were evaluated for objective response and operability. At surgery, patients were classified as exhibiting a complete/microscopic response, i.e. the complete disappearance or the presence of tumour cells only microscopically detectable (pathological response 1, PR1 patients;  $n = 6$ ), or macroscopic residual tumour (pathological response 2, PR2 patients;  $n = 3$ ). Mononuclear cell suspensions from LN and TDLN samples were obtained immediately after surgery, as described previously.<sup>21</sup> Briefly, tissue was mechanically disaggregated using a scalpel and needle followed by syringing through a 22-gauge needle under sterile conditions. PB lymphocytes (PBL) were obtained as mononuclear cells by standard density gradient centrifugation of heparinized blood, as described.<sup>23</sup>

### *Flow cytometry analysis*

Four-colour flow cytometry was performed using monoclonal antibodies (mAbs) to the following surface antigens: CD3, CD4, CD8, CD25, CD45RO, and human

leucocyte antigen (HLA)-DR (Beckman Coulter, Miami, FL), GITR (PharMingen, San Diego, CA), anti-Nrp1 antibody BDCA4 (Miltenyi Biotec, Bergisch-Gladbach, Germany), and Foxp3 (PCH101 clone; eBioscience, San Diego, CA). The mAbs were purchased as conjugates with the fluorescent dyes fluorescein isothiocyanate (FITC), phycoerythrin (PE), PE-Texas Red (ECD) and PE-Cyanin 5.1 (PC5), and appropriately combined to assess the cell subset of interest. Foxp3 had to be determined by intracellular staining.<sup>25</sup> To this end, cells were stained for surface antigens, washed and then fixed and permeabilized using the staining kit provided by eBioscience according to the manufacturer's instructions. With permeabilized lymphocytes, mAbs can give increased background fluorescence, possibly as the result of entry of free fluorochrome and/or mAb reactivity with charged or polar internal molecules, which cannot be correctly evaluated by the conventional isotype staining. Here we overcame this complication by first incubating cells with an eight-fold molar excess of unlabelled anti-Foxp3 mAb PCH101 clone to completely saturate the specific binding sites and finally with the FITC-conjugated anti-Foxp3 mAb. Flow cytometry was performed using a Beckman Coulter XL flow cytometer equipped for four-colour immunofluorescence. A minimum of 5000 cells of interest were acquired for each sample. Typically, this required at least 200 000 events to be acquired. List mode data were then analysed using Expo 32<sup>TM</sup> (Beckman Coulter) software. Purity of allophycocyanin (APC)-BDCA4 mAb-enriched cells (see below) was checked on a Becton Dickinson FACSCalibur (BD Biosciences, Mountain View, CA) using CELLQUEST software.

#### Cell isolation and culture

Purification of Nrp1<sup>+</sup> cells was performed as follows using an immunomagnetic microbead-based sorting technique (Miltenyi Biotec) according to the manufacturer's instructions. It was important to first exclude CD4<sup>+</sup> Nrp1<sup>+</sup> plasmacytoid dendritic cells (pDC), which are particularly abundant in LN.<sup>26</sup> To this end, LN cell suspensions were first enriched for T cells by double-positive selection using the PE-CD3 mAb and anti-PE multisort kit, and LS columns. Next, CD25<sup>high</sup> cells were purified by double-positive selection using the FITC-CD25 mAb and anti-FITC multisort kit and MS columns. Nrp1<sup>+</sup> cells were then purified by double-positive selection from CD25<sup>high</sup> and CD25<sup>-</sup> T cells by APC-BDCA4 mAb and anti-APC microbeads using MS columns. This strategy allowed a high enrichment of CD4<sup>+</sup> T cells, typically > 85% in CD25<sup>high</sup> Nrp1<sup>+</sup> and CD25<sup>high</sup> Nrp1<sup>-</sup> cell preparations, as the result of both the paucity of CD8<sup>+</sup> T cells in LN, typical CD4<sup>+</sup>:CD8<sup>+</sup> ratio > 5, and the absence of CD8<sup>+</sup> CD25<sup>high</sup> and CD8<sup>+</sup> Nrp1<sup>+</sup> T cells. These cells will hereafter be referred to as Nrp1<sup>+</sup> Treg and Nrp1<sup>-</sup> Treg.

The final purity of the Nrp1<sup>+</sup> Treg population used for functional and real-time reverse transcription-polymerase chain reaction (RT-PCR) assays was  $\geq 60\%$  while the Nrp1<sup>-</sup> Treg population was virtually devoid of Nrp1-staining cells. Attempts made to obtain CD4<sup>+</sup> CD25<sup>-</sup> Nrp1<sup>+</sup> T cells produced modest results in terms of yield and purity. Therefore, this cell subset was not assessed functionally. For comparison, Treg were also purified using a CD4<sup>+</sup> CD25<sup>+</sup> regulatory T-cell isolation kit (Miltenyi Biotec), according to the manufacturer's instructions. Purification of autologous monocytes to be used as APC was performed using CD14-coated microbeads (Miltenyi Biotec). To verify the proliferative responsiveness to polyclonal activation, cells were seeded in replicate wells in a standard U-bottomed, 96-well culture plate (Falcon, BD Biosciences) precoated overnight with a mixture of anti-CD3 and anti-CD28 (clone YTH913.12 1  $\mu\text{g/ml}$ ; Serotec Ltd, Oxford, UK). Phytohaemagglutinin (PHA; 1  $\mu\text{g/ml}$ ; Sigma, St Louis, MO) was also used and gave results that were essentially analogous to those obtained for CD3/CD28 stimulation. To verify the regulatory capacity on T-cell proliferation in response to polyclonal activation, the indicated numbers of autologous responder and suppressor cells were seeded in replicate wells in a standard U-bottomed, 96-well culture plate (Falcon) precoated overnight with a mixture of anti-CD3 and anti-CD28 (clone YTH913.12 1  $\mu\text{g/ml}$ , Serotec Ltd). PHA (1  $\mu\text{g/ml}$ ) was also used and gave results that were essentially analogous to those obtained for CD3/CD28 stimulation. Responder cells in inhibition experiments were autologous PBL devoid of CD25-expressing cells using microbeads directly coated with anti-CD25 mAb (Miltenyi Biotec). Incubation was carried out at 37° in a 5% CO<sub>2</sub> atmosphere for 5 days. For Transwell experiments, 24-well, flat-bottom plates (Falcon) were used. Autologous responder CD25-depleted PBL, and either Nrp1<sup>+</sup> Treg or Nrp1<sup>-</sup> Treg were plated in the lower and upper chamber of each Transwell, respectively. Unfractionated Treg served as control. To test the functional role of Nrp1 on Treg, preservative-free anti-Nrp1 mouse mAb M01, clone 3G6-2C5 (10  $\mu\text{g/ml}$ ) (Abnova Co., Taipei, Taiwan) was used.

In each proliferation assay, the response was assessed using the intracellular covalent coupling dye carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Eugene, OR). The staining procedure was essentially as described previously.<sup>27</sup> Briefly, responder cells were aseptically loaded with 0.2  $\mu\text{M}$  CFSE before plating. The number of cell divisions was quantified by MODFIT<sup>TM</sup>/CELL PROLIFERATION MODEL<sup>TM</sup> software.

#### Cytokine analysis

Cytokines were determined in the supernatants from cultured cells collected 48 hr after seeding cultures as for the

proliferation assays above. The presence of IL-2 and interferon- $\gamma$  (IFN- $\gamma$ ) in the supernatant of the stimulated cells was measured by multiplex enzyme-linked immunosorbent assay (ELISA) using the Pierce SearchLight technology (Pierce Boston Technology, Woburn, MA). Cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum and antibiotics (all from Gibco, New York, NY) in 96-well, U-bottom plates. PHA was used as a mitogen.

#### Real-time RT-PCR

Total RNA was extracted from Nrp1<sup>+</sup> Treg, Nrp1<sup>-</sup> Treg, and unfractionated Treg populations and from CD4<sup>+</sup> T cells devoid of CD25-expressing cells using Trizol according to the manufacturer's protocols (Invitrogen Life Technologies, Paisley, UK). Total RNA was eluted in diethylpyrocarbonate (DEPC)-treated water (0.01% DEPC) and stored at -80° until RT-PCR analysis. Nucleic acid concentrations were measured by spectrophotometry (Hewlett-Packard HP UV/VIS spectrophotometer 8450; Palo Alto, CA). Complementary DNA (cDNA) was synthesized using a QuantiTect<sup>®</sup> Reverse Transcription Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Foxp3 messenger RNA (mRNA) expression was quantified by sequence-specific fluorescence-based real-time PCR (QuantiTect<sup>®</sup> technology; QIAGEN, Valencia, CA) in relative quantitative assay using CD3 $\epsilon$  as reference for normalization. PCR was performed using QuantiTect<sup>®</sup> Gene Expression Assay for Foxp3 (Qiagen) and QuantiTect<sup>®</sup> Custom Assay for CD3 $\epsilon$  (Qiagen) as primer-probe sets, and QuantiTect<sup>®</sup> Probe PCR Kits as the master mix. The thermalcycler used for real-time amplifications was LightCycler (Roche, Mannheim, Germany). PCR data were analysed by RELATIVE QUANTIFICATION SOFTWARE (Roche) and expressed as the ratio of target gene (Foxp3) : reference gene (CD3 $\epsilon$ ).

#### Statistics

An analysis of variance (ANOVA) followed by Dunnett's multiple comparison test was used for the analysis of the statistical significance among more than two groups. Student's *t*-test was used for the analysis of the statistical significance between two groups.

#### Results

##### Frequency and Foxp3 expression of Nrp1<sup>+</sup> CD4<sup>+</sup> T cells in LN and PB

The brightness of CD25 staining is correlated with Treg function in humans.<sup>4</sup> We first determined Nrp1 expression in relation to CD25 expression in LN and PB. To

**Table 1.** Frequency of Nrp1<sup>+</sup> cells among CD4<sup>+</sup> T cells expressing different levels of CD25 in LN and PB

|    | CD25 <sup>neg</sup> | CD25 <sup>int</sup> | CD25 <sup>high</sup> |
|----|---------------------|---------------------|----------------------|
| LN | 0.27 $\pm$ 0.46     | 2.1 $\pm$ 1.77      | 8.67 $\pm$ 4.30      |
| PB | 0.39 $\pm$ 0.29     | 0.50 $\pm$ 0.76     | 0.42 $\pm$ 0.56      |

this end, cells were stained with mAbs to Nrp1, CD3, CD25 and either CD4 or CD8. Three CD4<sup>+</sup> T-cell subsets were defined according to the CD25 expression level, namely CD25<sup>neg</sup>, CD25<sup>int</sup> and CD25<sup>high</sup>, as described elsewhere.<sup>24</sup> As summarized in Table 1, the frequency of Nrp1<sup>+</sup> cells increased in step with CD25 expression level in LN. In PB, the frequency of Nrp1<sup>+</sup> cells did not correlate with the CD25 expression level and was lower than in LN (Table 1). No CD8<sup>+</sup> T cell stained positive for Nrp1 in LN and PB (not shown). Next, we investigated the expression of Foxp3 in Nrp1<sup>+</sup> CD4<sup>+</sup> T cells in relation to CD25 expression. Measures of Nrp1 expression in four-colour flow cytometry may be biased by the presence of Nrp1<sup>+</sup> CD4<sup>+</sup> pDCs, which are particularly abundant in LN. To overcome this technical limitation, cells were stained with mAbs to Foxp3, Nrp1, CD3 and CD25, and CD3/side-scatter dot plots were gated so as to include only T cells in the analysis. The absence of CD8<sup>+</sup> Nrp1<sup>+</sup> cells, the marginal presence of CD25 expression by CD8 cells and the low proportion of CD8<sup>+</sup> T cells in LN, allow this strategy to indicate Foxp3 and CD25 expression in Nrp1<sup>+</sup> CD4<sup>+</sup> T cells. Figure 1(a) shows the relationship between Nrp1 and CD25 expression in CD4<sup>+</sup> T cells in LN and PB (top and bottom plot, respectively). Figure 1(b) (top plots) exemplifies the gate used to assess Foxp3/CD25 coexpression in Nrp1<sup>+</sup> CD4<sup>+</sup> T cells from a representative LN sample. About half of Nrp1<sup>+</sup> CD4<sup>+</sup> T cells coexpressed Foxp3 and CD25 (Fig. 1b, top plot) or lacked both markers, while minor numbers of cells expressed either Foxp3 or CD25. The same procedure applied to PB (Fig. 1b, bottom plots) showed that Nrp1 was expressed by a marginal number of T cells (bottom left plot) that were Foxp3<sup>-</sup> and expressed CD25 at a low level (Fig. 1b, bottom right plot). Thus, we concluded that Nrp1 identified T cells that were potentially endowed with regulatory properties in LN but not in PB and we focused on Nrp1<sup>+</sup> T cells in LN to investigate the co-expression of various markers for Treg. To this end, cells extracted from LN samples were stained with mAbs to CD3, Nrp1, CD25 and either Foxp3, GITR HLA-DR, or CD45RO. We found that Foxp3, GITR and HLA-DR tended to be preferentially expressed by Nrp1<sup>+</sup> cells within each CD4<sup>+</sup> T-cell subset defined by the intensity of CD25 staining (Table 2). The CD45RO expression level showed no clear relationship with Nrp1 expression (Table 2).

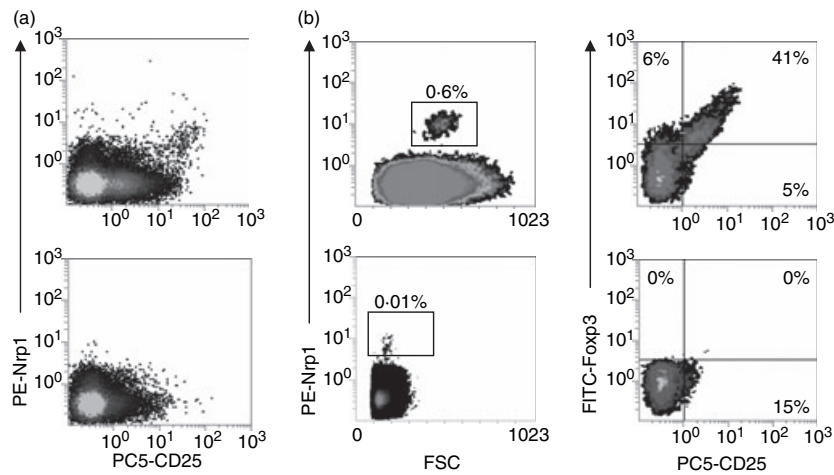


**Figure 1.** Nrp1 expression on CD4<sup>+</sup> T cells in human LN and PB. (a) Nrp1 expression in relation to CD25 staining intensity in CD4<sup>+</sup> T cells in LN (top plot) and PB (bottom plot). Most Nrp1<sup>+</sup> cells in LN are CD25<sup>high</sup> whereas in PB Nrp1 expression is essentially restricted to CD25<sup>+</sup> cells. (b) Gating of Nrp1<sup>+</sup> T cells in LN (top left plot) and PB (bottom left plot) and assessment of their Foxp3 and CD25 expression in LN (top right plot) and PB (bottom right plot). Quadrants are drawn based on fluorescence signal generated by samples stained with isotype-matched control antibody for PC5-CD25 and an eightfold molar excess of unlabelled anti-Foxp3 mAb followed by FITC-conjugated anti-Foxp3 mAb for FITC-Foxp3. Numbers in plots represent the percentage of cells in each region.

**Table 2.** Phenotypic characterization of CD4<sup>+</sup> Nrp1<sup>−</sup> and CD4<sup>+</sup> Nrp1<sup>+</sup> T cells expressing different levels of CD25 in LN

|                     | CD25 <sup>neg</sup> |                   | CD25 <sup>int</sup> |                   | CD25 <sup>high</sup> |                   |
|---------------------|---------------------|-------------------|---------------------|-------------------|----------------------|-------------------|
|                     | Nrp1 <sup>−</sup>   | Nrp1 <sup>+</sup> | Nrp1 <sup>−</sup>   | Nrp1 <sup>+</sup> | Nrp1 <sup>−</sup>    | Nrp1 <sup>+</sup> |
| Foxp3 <sup>+</sup>  | 9.5 ± 4.8           | 26.0 ± 21.1       | 46.9 ± 19.1         | 72.1 ± 16.0       | 87.9 ± 6.8           | 90.3 ± 10.8       |
| GITR <sup>+</sup>   | 5.7 ± 5.6           | 11.1 ± 6.9        | 12.3 ± 9.7          | 37.6 ± 21.1       | 35.1 ± 23.6          | 91.1 ± 7.8        |
| DR <sup>+</sup>     | 4.1 ± 0.6           | 8.6 ± 1.1         | 4.6 ± 0.2           | 9.3 ± 0.5         | 16.0 ± 2.0           | 85 ± 3.1          |
| CD45RO <sup>+</sup> | 59.2 ± 12.4         | 79.3 ± 10.8       | 85.4 ± 9.3          | 88.9 ± 8.0        | 83.1 ± 7.2           | 93.1 ± 2.2        |

### Nrp1<sup>+</sup> Treg proliferate poorly and suppress proliferation of autologous T cells *in vitro*

To address the functional significance of Nrp1 on CD4<sup>+</sup> T cells, Nrp1<sup>+</sup> Treg and Nrp1<sup>−</sup> Treg were immunomagnetically sorted and analysed for their ability to respond to mitogenic stimulation *in vitro*. Both Nrp1<sup>+</sup> Treg and Nrp1<sup>−</sup> Treg were hyporesponsive to polyclonal stimulation; this was similar to the unfractionated Treg population used as comparison (Fig. 2). Titration of increasing numbers of Nrp1<sup>+</sup> Treg into cultures with a fixed dose of responding CD25-depleted autologous PBL led to a marked decrease in proliferation of the latter (Fig. 3). Nrp1<sup>−</sup> Treg and unfractionated Treg populations were comparatively less efficient, which was more evident at the lowest responder : suppressor ratio (Fig. 3). As a control, it was shown that titration of the same dose of CD4<sup>+</sup> CD25<sup>−</sup> T cells did not affect the degree of proliferation (Fig. 3).

### Suppression of cytokine production

The effect of Nrp1<sup>+</sup> Treg and Nrp1<sup>−</sup> Treg on cytokine secretion in coculture conditions (suppressor : responder

ratio 1 : 1) was examined next. An unfractionated Treg population was used for comparison. We analysed supernatant collected from the cultures illustrated in Fig. 3 for levels of IL-2 and IFN- $\gamma$ . Nrp1<sup>+</sup> Treg, Nrp1<sup>−</sup> Treg and unfractionated Treg populations suppressed cytokine production by responder cells equally (Fig. 4).

### The mAbs to Nrp1 do not block the suppressive activity of Nrp1<sup>+</sup> Treg

Having demonstrated that Nrp1 expression is associated with a strong suppressive activity, we next asked if there could be a direct interaction between Nrp1 on the cell surface and the suppressive activity. To this end, we analysed the influence of mAb to Nrp1 on the inhibitory activity of Nrp1<sup>+</sup> Treg. Purified Nrp1<sup>+</sup> Treg were cocultured with responder CD25-depleted PBL in the presence of 20  $\mu$ g/ml anti-Nrp1 mAb M01. Alternatively, purified Nrp1<sup>+</sup> Treg were preincubated with anti-BDCA4 mAb to saturate all antigenic sites, washed and cocultured with responder autologous CD25-depleted PBL (suppressor : responder ratio 1 : 10). This different strategy was dictated by the presence of preservative in the BDCA4 preparation, which strongly inhibited T-cell proliferation;

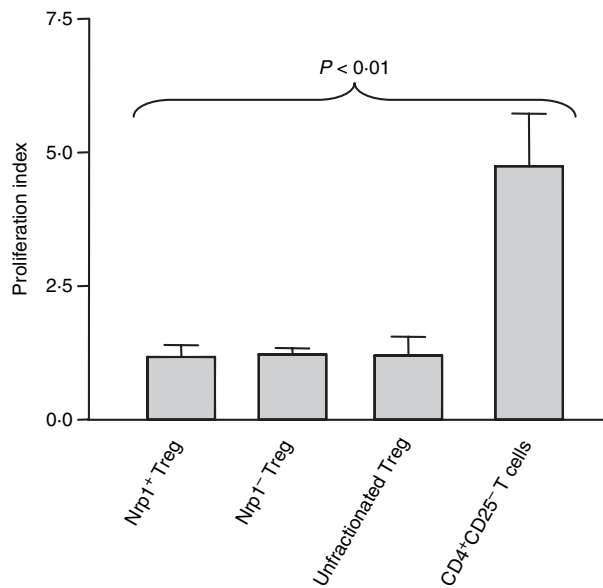


**Figure 2.** Responsiveness to polyclonal stimulation of unfractionated Treg, Nrp1<sup>+</sup> Treg, and Nrp1<sup>-</sup> Treg from LN. The proliferative responsiveness to polyclonal stimulation of the unfractionated Treg population and Treg subset defined by Nrp1 coexpression (all  $5 \times 10^4$  cells/well) isolated with marker-specific antibodies in combination with immunomagnetic beads as described in Materials and methods was compared to that of control CD4<sup>+</sup> CD25<sup>-</sup> T cells. Cells were aseptically loaded with CFSE as described in Materials and methods and the proliferative response (proliferation index) was assessed on day 5 by ModFit<sup>TM</sup>/CELL PROLIFERATION MODEL<sup>TM</sup> software. Mean values ( $\pm$  SD) obtained in four separate experiments are reported. Significance was assessed by ANOVA followed by Dunnet's multiple comparison test.

Fig. 5 shows that no treatment interfered with the suppressor function of Nrp1<sup>+</sup> Treg.

### Foxp3 message expression

The transcription factor Foxp3 was shown to be required for the suppressive activity of Treg in mice and humans.<sup>28</sup> To further establish the regulatory nature of Nrp1<sup>+</sup> Treg, we assessed Foxp3 mRNA expression in the Nrp1<sup>+</sup> Treg and Nrp1<sup>-</sup> Treg from LN using real-time quantitative RT-PCR. The amount of Foxp3 mRNA produced by Nrp1<sup>+</sup> Treg was  $\sim$  twofold higher than that produced by Nrp1<sup>-</sup> Treg. Consistently, the level of Foxp3 message in the unfractionated Treg population was intermediate between the results from Nrp1<sup>+</sup> Treg and Nrp1<sup>-</sup> Treg. The lowest level of Foxp3 transcript was found in CD4<sup>+</sup> CD25<sup>-</sup> T cells (Fig. 6).

### Modulation of Treg and Nrp1<sup>+</sup> Treg frequency in TDLN by preoperative CR in CC patients

The influence of preoperative CR on the Treg percentage within the CD4<sup>+</sup> T-cell population and in relation to the

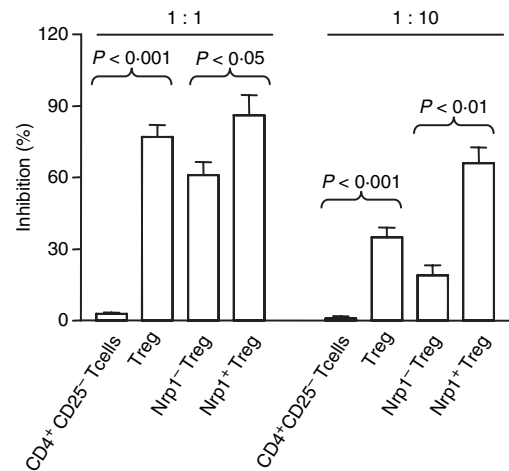


**Figure 3.** Suppressive activity of unfractionated Treg, Nrp1<sup>+</sup> Treg, and Nrp1<sup>-</sup> Treg from LN on mitogen-driven PBL proliferation. The functional activity of immunomagnetically purified Nrp1<sup>+</sup> Treg, Nrp1<sup>-</sup> Treg, unfractionated Treg population and CD4<sup>+</sup> CD25<sup>-</sup> T cells as a control was tested in a proliferation assay using CFSE-labelled autologous responder PBL immunomagnetically depleted of CD25-expressing cells. Responder cells ( $5 \times 10^4$ /well) were cultured in the presence of suppressor cells at 1:1 and 1:10 suppressor:responder ratio. The proliferative response was assessed on day 5 by computing CFSE halving by ModFit<sup>TM</sup>/CELL PROLIFERATION MODEL<sup>TM</sup> software. Mean values ( $\pm$ SD) obtained in five separate experiments are reported. Significance was assessed by Student's *t*-test.

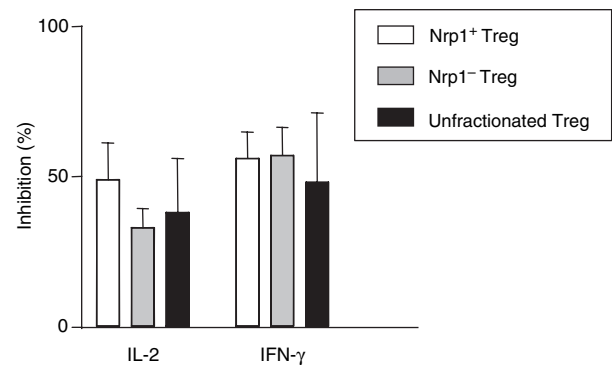


**Figure 4.** Unfractionated Treg and Treg subsets defined by Nrp1 expression from LN suppress the secretion of IL-2 and IFN- $\gamma$  by co-cultured CD25-depleted autologous PBL. Culture supernatants from the same cultures described in Fig. 3 were collected at day 2 and cytokines were determined by multiplex ELISA. Data are expressed as per cent variation of cytokine secretion of CD25-depleted autologous PBL ( $5 \times 10^4$ /well). Mean values ( $\pm$ SD) obtained in three separate experiments are reported.

pathological response in TDLN is shown in Fig. 7(a) (inset), respectively. The Treg percentage in TDLN from untreated and CR-treated patients appeared similar (Fig. 7a). However, subdividing the CR-treated patients according to the pathological response we observed a significant drop of Treg percentage associated with tumour

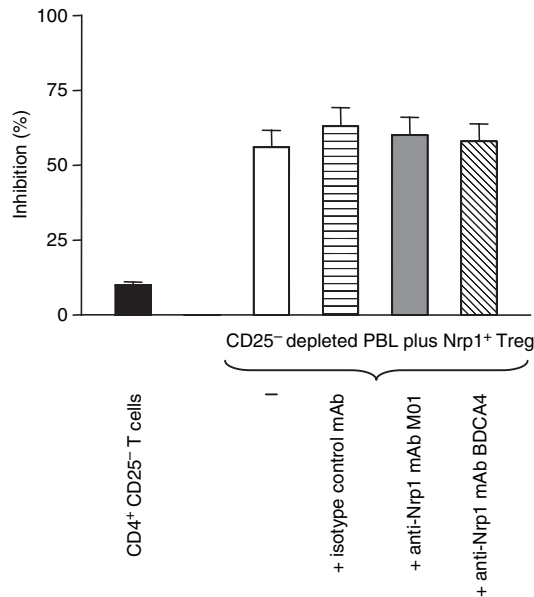


**Figure 5.** Nrp1 is not required for the suppressive activity of Nrp1<sup>+</sup> Treg. CFSE-labelled autologous responder CD25-depleted PBL were cocultured with Nrp1<sup>+</sup> Treg in the presence of 20 µg/ml anti-Nrp1 M01 mAb or mouse immunoglobulin as control. Alternatively, Nrp1<sup>+</sup> Treg were first preincubated with anti-Nrp1 BDCA4 mAb, washed and mixed with CFSE-labelled autologous responder CD25-depleted PBL. The proliferative response was assessed on day 5 by computing CFSE halving by ModFit<sup>TM</sup>/CELL PROLIFERATION MODEL<sup>TM</sup> software. Mean values (±SD) obtained in two separate experiments are reported.

mass reduction or disappearance (PR1 patients, Fig. 7a inset). The percentage of Nrp1<sup>+</sup> cells within the Treg population tended to be lowered by CR (Fig. 7b) but the highest reduction occurred in patients in which CR removed most of the tumour mass (PR1 patients, Fig. 7b inset). Thus, Treg and Nrp1<sup>+</sup> Treg reduction correspond to a favourable response to CR in CC.

## Discussion

The main goal of this study was the identification of the phenotypic and functional profiles of human Treg expressing Nrp1. Following the original observations that Nrp1 is involved in axonal guidance, promotion of angiogenesis and cell migration,<sup>11</sup> Nrp1 was later identified on human and murine CD4<sup>+</sup> T cells,<sup>13–15</sup> and shown to be involved in the functional control of these cells. Data were conflicting, however, as Nrp1 appeared to be involved in the initiation of the primary immune response in humans<sup>13</sup> and in immunosuppression in mice.<sup>14,15</sup> Here we present the first evidence that in humans Nrp1 expression identifies a Treg subset, at least in LN. When we examined the suppressive activity of Nrp1<sup>+</sup> Treg, we found that they share the same mecha-

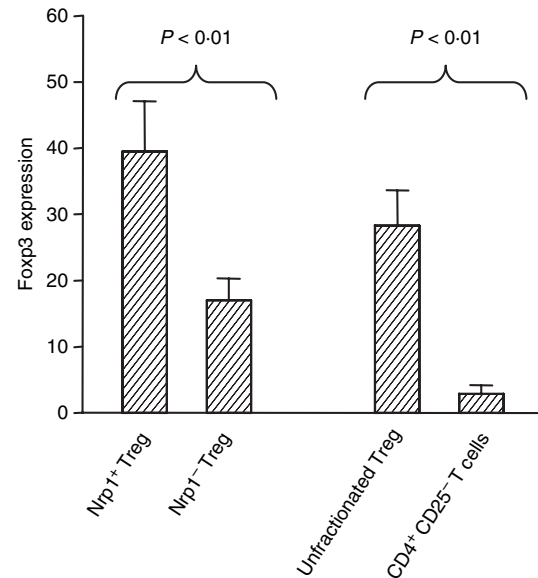


**Figure 6.** Foxp3 message in unfractionated Treg, Nrp1<sup>+</sup> Treg, and Nrp1<sup>-</sup> Treg from LN. RNA from unfractionated Treg and Treg subsets defined by Nrp1 expression was prepared as described in Materials and methods. CD4<sup>+</sup> CD25<sup>-</sup> T cells served as control. The cDNA was tested for Foxp3 expression by real-time RT-PCR using CD3ε mRNA expression as reference for normalization. Measurements were performed in triplicates and results are presented as Foxp3 expression relative to CD3ε. Representative data (averages and SD) from three separate experiments are shown.

nisms of suppression of classical Treg, i.e. CD4<sup>+</sup> CD25<sup>high</sup> T cells: they do not proliferate upon mitogenic stimulation, they inhibit the proliferation of autologous T cells by cell-to-cell contact, and the suppressive activity is accompanied by an inhibition of cytokine production. However, although the mechanisms involved in the suppression appear identical, Nrp1<sup>+</sup> Treg are more potent than their Nrp1<sup>-</sup> counterparts. To elucidate the reasons underlying the superior suppressive potency, we examined the phenotypic profile of Nrp1<sup>+</sup> Treg. There is evidence that strong HLA-DR and GITR expression represents the molecular signature of a particularly potent highly activated human Treg fraction.<sup>5,28,29</sup> Most Nrp1<sup>+</sup> Treg expressed HLA-DR and GITR, indicating that Nrp1 expression correlates with activation status.

In addition to Nrp1<sup>+</sup> Treg, LN contained a small fraction of Nrp1<sup>+</sup> CD4<sup>+</sup> T cells expressing CD25 at an intermediate level, and even lacking CD25 expression. Both Nrp1<sup>+</sup> CD25<sup>int</sup> and Nrp1<sup>+</sup> CD25<sup>neg</sup> T cells preferentially expressed Foxp3 and GITR compared to their Nrp1<sup>-</sup> counterparts. These phenotypic features are compatible with those of the regulatory subsets that are capable of expansion following activation described earlier,<sup>9,10</sup> posing the possibility that these cells are enhancing CD25 expression to become Nrp1<sup>+</sup> CD25<sup>high</sup> T cells, i.e. Nrp1<sup>+</sup> Treg. Unfortunately, because of the technical limitations

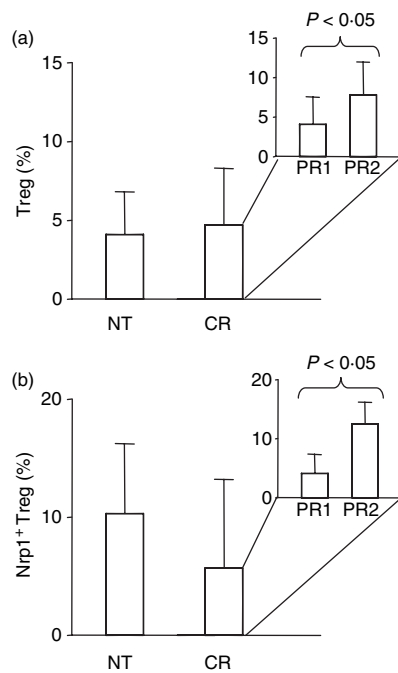


**Figure 7.** Treg and Nrp1<sup>+</sup> Treg in TDLN are modulated by preoperative chemoradiation therapy in CC patients in direct relationship with pathological response. TDLN-derived cells were stained with FITC anti-CD4, PE anti-Nrp1, ECD anti-CD3, and PE-Cy5 anti-CD25 mAbs. (a) CR did not modify the percentage of Treg within CD4<sup>+</sup> T cells. (b) CR decreased Nrp1<sup>+</sup> cells within Treg, although not significantly. Following CR, a significant reduction of Treg and Nrp1<sup>+</sup> cells within Treg was seen in patients with a complete/near complete disappearance of the primary tumour mass (PR1) compared to patients with a macroscopic residual tumour (PR2) (insets in a and b, respectively). Mean values ( $\pm$ SD).

inherent in magnetic microbead cell sorting, it was not possible to formally prove or disprove this possibility.

Circulating Nrp1<sup>+</sup> CD4<sup>+</sup> T cells did not display the phenotypic hallmarks of Treg and, although their exceedingly low level hampered functional studies, one can assume that they are not suppressive. This conclusion supports the earlier study by Tordjman *et al.*,<sup>13</sup> which described how Nrp1 expressed by human circulating CD4<sup>+</sup> T cells helps to establish the immunological synapse between T cells and DC and stimulates T-cell activation. At this point it is important to stress the disagreement among earlier reports<sup>13–15</sup> in defining the significance of Nrp1 expression on CD4<sup>+</sup> T cells. We believe that the present data may provide a possible explanation for the discrepancy. We suggest that the non-regulatory Nrp1<sup>+</sup> CD4<sup>+</sup> T cells in PB that were found here correspond to the circulating Nrp1<sup>+</sup> CD4<sup>+</sup> T cells described in the Tordjman *et al.* study,<sup>13</sup> whereas the Nrp1<sup>+</sup> Treg found in LN correspond to the suppressive splenic Nrp1<sup>+</sup> Treg described in the Bruder *et al.* study.<sup>14</sup> Incidentally, this raises the intriguing question as to what extent differences in the anatomical source of the cell

population under scrutiny may underlie the differences between murine and human immune systems.

Next, we tested whether the functional property of Nrp1<sup>+</sup> Treg depended directly upon the presence of Nrp1 on the cell surface. This seemed unlikely, inasmuch as Nrp1 mediates homophilic interaction in the immune system<sup>13</sup> and there was virtually no detectable Nrp1 on the CD25-depleted responder cells used as responders in functional assays. Nevertheless, we tested this possibility in function-blocking experiments and found that coculture suppression proceeded in the presence of anti-Nrp1 mAb. These data imply that Nrp1 is essentially a marker of activated Treg with no direct involvement in the suppressive capability, although it cannot be ruled out that other anti-Nrp1 mAbs effective in different experimental systems<sup>13</sup> can also neutralize Nrp1<sup>+</sup> Treg activity. To add to the complexity, the same anti-Nrp1 mAb BDCA4 that was ineffective in our experiments has been shown to suppress the response of pDC to various activators,<sup>30</sup> suggesting that this antibody can bind to a functionally significant moiety of Nrp1 but that the effect is cell-type dependent. However, that Nrp1 identifies a powerful Treg subset but its blockade has no detectable functional effect is reminiscent of an analogous phenomenon described in an earlier study in which HLA-DR expression identified a powerful Treg subset but was not implicated in the suppressor activity.<sup>5</sup>

The relationship between Nrp1<sup>+</sup> CD4<sup>+</sup> T cells in LN and PB remains to be elucidated. Circulating Nrp1<sup>+</sup> CD4<sup>+</sup> non-regulatory T cells may be on the way to becoming Treg upon entering lymphoid organs, where surface Nrp1 would enable interactions with pDC through homophilic binding.<sup>31</sup> Thus, in a microenvironment that is rich in pDC, where self-peptide recognition is continuous, Nrp1 may favour the homeostatic maintenance of Treg. In that regard, studies in mice have shown that trafficking pDC to LN correlates with tolerance, and Foxp3 expression in CD4<sup>+</sup> T cells.<sup>32</sup>

In the second part of the study, we focused on the presence of Nrp1<sup>+</sup> Treg in TDLN. Lymphocytes are mobile cells and during transit through the TDLN they encounter tumour antigens derived from the primary tumour mass in association with antigen-presenting cells and become functionally competent. Thus, the distribution of lymphocyte subsets in TDLN is a reflection of the interaction between tumour and immune system. We previously showed<sup>23</sup> that the efficacy of preoperative CR in CC patients correlated with a switch from a resting/naive phenotype to an activated/effector phenotype and a concomitant Treg frequency decrease in TDLN. With this background, we evaluated whether CR affected Nrp1<sup>+</sup> Treg in TDLN. Confirming and extending earlier data, we observed that Nrp1<sup>+</sup> Treg numbers fell sharply in relationship with CR-induced reduction of tumour mass, their diminution being even more evident than that of

the whole Treg population. At least two not mutually exclusive mechanisms may be invoked to explain why a complete or near complete destruction of primary tumour mass associates with suppressive cell drop. First, killing tumour cells may obviously eliminate tumour-derived factors, including immunosuppressive cytokines to which TDLN are most intensively exposed.<sup>33</sup> Second, dying tumour cells may have induced inflammatory signals, possibly via an enhanced apoptotic/necrotic tumour cell uptake by DC,<sup>34</sup> pushing T cells in the immunological synapse to preferentially undergo differentiation towards an activation/effector phenotype rather than a suppressive phenotype.

In summary, this study shows the existence of a previously unrecognized population of Treg in human LN that expresses Nrp1 and is able to mediate a potent contact-dependent suppression *in vitro*. We also show that Nrp1-expressing CD4<sup>+</sup> T cells in PB are not regulatory. Lastly, we demonstrate that successful CR in CC is associated with a low Nrp1<sup>+</sup> Treg frequency in TDLN.

## References

- Zwar TD, Van Driel IR, Gleeson PA. Guarding the immune system: suppression of autoimmunity by CD4CD25 immunoregulatory T cells. *Immunol Cell Biol* 2006; **84**:487–501.
- Dejaco C, Duftner C, Grubeck-Loebenstien B, Schirmer M. Imbalance of regulatory T cells in human autoimmune diseases. *Immunology* 2006; **117**:289–300.
- Zou W. Regulatory T cells, tumour immunity and immunotherapy. *Nat Rev Immunol* 2006; **6**:295–307.
- Baecher-Allan C, Brown JA, Freeman GJ, Hafler DA. CD4<sup>+</sup> CD25<sup>high</sup> regulatory cells in human peripheral blood. *J Immunol* 2001; **167**:1245–53.
- Baecher-Allan C, Wolf E, Hafler DA. MHC class II expression identifies functionally distinct human regulatory T cells. *J Immunol* 2006; **176**:4622–31.
- Wing K, Suri-Payer E, Rudin A. CD4<sup>+</sup> CD25<sup>+</sup>-regulatory T cells from mouse to man. *Scand J Immunol* 2005; **62**:1–15.
- Yi H, Zhen Y, Jiang L, Zheng J, Zhao Y. The phenotypic characterization of naturally occurring regulatory CD4<sup>+</sup> CD25<sup>+</sup> T cells. *Cell Mol Immunol* 2006; **3**:189–95.
- Chen W, Jin W, Hardegen N, Li KJ, Li L, Marinos N, McGrady G, Wahl SM. Conversion of peripheral CD4<sup>+</sup> CD25<sup>−</sup> naive T cells to CD4<sup>+</sup> CD25<sup>+</sup> regulatory T cells by TGF- $\beta$  induction of transcription factor Foxp3. *J Exp Med* 2003; **198**:1875–86.
- Zelenay S, Lopes-Carvalho T, Caramalho I, Moraes-Fontes MF, Rebelo M, Demengeot J. Foxp3<sup>+</sup> CD25<sup>−</sup> CD4 T cells constitute a reservoir of committed regulatory cells that regain CD25 expression upon homeostatic expansion. *Proc Natl Acad Sci USA* 2005; **102**:4091–6.
- Pan Q, Chantry Y, Liang WC *et al*. Blocking neuropilin-1 function has an additive effect with anti-VEGF to inhibit tumor growth. *Cancer Cell* 2007; **11**:53–67.
- Chen H, He Z, Bagri A, Tessier-Lavigne M. Semaphorin–neuropilin interactions underlying sympathetic axon responses to class III semaphorins. *Neuron* 1998; **21**:1283–90.
- Kikutani H, Kumanogoh A. Semaphorins in interactions between T cells and antigen-presenting cells. *Nat Rev Immunol* 2003; **3**:159–67.
- Tordjman R, Lepelletier Y, Lemarchandel V, Cambot M, Gaulard P, Hermine O, Romeo PH. A neuronal receptor, neuropilin-1, is essential for the initiation of the primary immune response. *Nat Immunol* 2002; **3**:477–82.
- Bruder D, Probst-Kepper M, Westendorf AM *et al*. Neuropilin-1: a surface marker of regulatory T cells. *Eur J Immunol* 2004; **34**:623–30.
- Delgado M, Chorny A, Gonzalez-Rey E, Ganea D. Vasoactive intestinal peptide generates CD4<sup>+</sup> CD25<sup>+</sup> regulatory T cells *in vivo*. *J Leukoc Biol* 2005; **78**:1327–38.
- Lim AY, Price P, Beilharz MW, French MA. Cell surface markers of regulatory T cells are not associated with increased forkhead box p3 expression in blood CD4<sup>+</sup> T cells from HIV-infected patients responding to antiretroviral therapy. *Immunol Cell Biol* 2006; **84**:530–6.
- Ndhlovu LC, Takeda I, Sugamura K, Ishii N. Expanding role of T-cell costimulators in regulatory T-cell function: recent advances in accessory molecules expressed on both regulatory and non-regulatory T cells. *Crit Rev Immunol* 2004; **24**:251–66.
- Santin AD, Hermonat PL, Ravaggi A *et al*. Development, characterization and distribution of adoptively transferred peripheral blood lymphocytes primed by human papillomavirus 18 E7-pulsed autologous dendritic cells in a patient with metastatic adenocarcinoma of the uterine cervix. *Eur J Gynaecol Oncol* 2000; **21**:17–23.
- Santin AD, Bellone S, Palmieri M, Roman JJ, Pecorelli S, Parham GP, Cannon MJ. Induction of tumor-specific cytotoxicity in tumor infiltrating lymphocytes by HPV16 and HPV18 E7-pulsed autologous dendritic cells in patients with cancer of the uterine cervix. *Gynecol Oncol* 2003; **89**:271–80.
- Rose PG, Eifel PJ. Combined radiation therapy and chemotherapy for carcinoma of the cervix. *Cancer J* 2001; **7**:86–94.
- Jurado M, Martinez-Monge R, Garcia-Foncillas J, Azinovic I, Aristu J, Lopez-Garcia G, Brugarolas A. Pilot study of concurrent cisplatin, 5-fluorouracil, and external beam radiotherapy prior to radical surgery +/- intraoperative electron beam radiotherapy in locally advanced cervical cancer. *Gynecol Oncol* 1999; **74**:30–7.
- Mariagrazia D, Anna F, Gabriella F, Francesco F, Daniela S, Giuseppe D, Alessio M, Giovanni S. Preoperative chemoradiotherapy in locally advanced cervical cancer: long-term outcome and complications. *Gynecol Oncol* 2005; **99**:S166–70.
- Fattorossi A, Battaglia A, Ferrandina G, Coronetta F, Legge F, Salutati V, Scambia G. Neoadjuvant therapy changes the lymphocyte composition of tumor-draining lymph nodes in cervical carcinoma. *Cancer* 2004; **100**:1418–28.
- Battaglia A, Ferrandina G, Buzzonetti A *et al*. Lymphocyte populations in human lymph nodes. Alterations in CD4<sup>+</sup> CD25<sup>+</sup> T regulatory cell phenotype and T-cell receptor V $\beta$  repertoire. *Immunology* 2003; **110**:304–12.
- Roncador G, Brown PJ, Maestre L *et al*. Analysis of FOXP3 protein expression in human CD4<sup>+</sup> CD25<sup>+</sup> regulatory T cells at the single-cell level. *Eur J Immunol* 2005; **35**:1681–91.
- Dzionek A, Fuchs A, Schmidt P, Cremer S, Zysk M, Miltenyi S, Buck DW, Schmitz J. BDCA-2, BDCA-3, and BDCA-4: three

- markers for distinct subsets of dendritic cells in human peripheral blood. *J Immunol* 2000; **165**:6037–46.
- 27 Fattorossi A, Battaglia A, Malinconico P *et al.* Constitutive and inducible expression of the epithelial antigen MUC1 (CD227) in human T cells. *Exp Cell Res* 2002; **280**:107–18.
- 28 Yagi H, Nomura T, Nakamura K *et al.* Crucial role of FOXP3 in the development and function of human CD25<sup>+</sup> CD4<sup>+</sup> regulatory T cells. *Int Immunol* 2004; **16**:1643–56.
- 29 McHugh RS, Whitters MJ, Piccirillo CA, Young DA, Shevach EM, Collins M, Byrne MC. CD4<sup>+</sup> CD25<sup>+</sup> immunoregulatory T cells: gene expression analysis reveals a functional role for the glucocorticoid-induced TNF receptor. *Immunity* 2002; **16**:311–23.
- 30 Grage-Griebenow E, Löseke S, Kauth M, Gehlar K, Zawatzky R, Bufe A. Anti-BDCA-4 (neuropilin-1) antibody can suppress virus-induced IFN- $\alpha$  production of plasmacytoid dendritic cells. *Immunol Cell Biol* 2007; **85**:383–90.
- 31 Tang Q, Bluestone JA. Plasmacytoid DCs and T(reg) cells: casual acquaintance or monogamous relationship? *Nat Immunol* 2006; **7**:551–3.
- 32 Ochando JC, Homma C, Yang Y *et al.* Alloantigen-presenting plasmacytoid dendritic cells mediate tolerance to vascularized grafts. *Nat Immunol* 2006; **7**:652–62.
- 33 Cochran AJ, Huang RR, Lee J, Itakura E, Leong SP, Essner R. Tumour-induced immune modulation of sentinel lymph nodes. *Nat Rev Immunol* 2006; **6**:659–70.
- 34 Ronchetti A, Rovere P, Iezzi G *et al.* Immunogenicity of apoptotic cells *in vivo*: role of antigen load, antigen-presenting cells, and cytokines. *J Immunol* 1999; **163**:130–6.