

# Metastatic tumour cells favour the generation of a tolerogenic milieu in tumour draining lymph node in patients with early cervical cancer

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## Abstract

**Objective** We compared the immune system state in metastatic tumour draining lymph nodes (mTDLN) and metastasis free TDLN (mfTDLN) in 53 early stage cervical cancer patients to assess whether the presence of metastatic tumour cells worsen the balance between an efficacious anti-tumour and a tolerogenic microenvironment.

**Methods** The immune system state was measured by immunophenotypic and functional assessment of suppressor and effector immune cell subsets.

**Results** Compared to mfTDLN, mTDLN were significantly enriched in CD4<sup>+</sup>Foxp3<sup>+</sup> regulatory T cells (Treg), which, in addition, exhibited an activated phenotype (HLA-DR<sup>+</sup> and CD69<sup>+</sup>). Treg in mTDLN were also significantly enriched in neuropilin-1 (Nrp1) expressing cells, a subset particularly potent in dampening T cell responses. mTDLN tended to be enriched in a population of CD8<sup>+</sup>Foxp3<sup>+</sup>T cells (operationally defined as CD8<sup>+</sup>Treg) that showed a suppressor potency similar to Treg under the same experimental conditions. Plasmacytoid dendritic cells (pDC) and

myeloid DC (mDC) generally show distinct roles in inducing T cell tolerance and activation, respectively. In line with the excess of suppressor T cells, the ratio pDC to mDC was significantly increased in mTDLN. Immunohistochemical testing showed that metastatic tumour cells produced the vascular endothelial growth factor, a natural ligand for Nrp1 expressed on the cell surface of Nrp1<sup>+</sup>Treg and pDC, and therefore a potential mediator by which tumour cells foster immune privilege in mTDLN. Consistent with the overall tolerogenic profile, mTDLN showed a significant Tc2 polarisation and tended to contain lower numbers of CD45RA<sup>+</sup>CD27<sup>-</sup> effector memory CD8<sup>+</sup>T cells.

**Conclusions** The increased recruitment of suppressor type cells concomitant with the scarcity of cytotoxic type cells suggests that in mTDLN the presence of tumour cells could tip the balance against anti-tumour immune response facilitating the survival of metastatic tumour cells and possibly contributing to systemic tolerance.

**Keywords** Cervical cancer · Tumour draining lymph nodes · Tumour metastasis · Immune state · Regulatory T cells · Regulatory CD8<sup>+</sup>Foxp3<sup>+</sup> cells

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## Introduction

The immune system allows some stimuli to provoke immune responses and consequently induces immunity, and prevents others from doing so and consequently induces tolerance. With respect to neoplasms, immunity or tolerance means the success or failure, respectively, of the immune system to eliminate a tumour. Thus, as tumour escapes from immune destruction, it is obvious that it has succeeded in inducing tolerance. In tumour immunology, the presentation of tumour antigens to naïve T cells takes

place in regional lymph nodes, thereafter referred to as tumour draining lymph nodes (TDLN), in principle the optimal environment for the generation of an effective anti-tumour immune response [10, 34]. However, TDLN are under the influence of tolerogenic tumour-derived factors such as cytokines and other bioactive molecules produced by tumour cells and their associated leukocytes in the primary tumour site [10, 34, 55]. A T cell tolerance rather than activation is therefore induced to prevent the immune attack and facilitate tumour progression. We reasoned that tolerance should be mostly evident in metastatic TDLN (mTDLN) that are also exposed to tolerogenic factors locally produced by tumour cells. Moreover, as most tumours metastasize first in TDLN, which can be considered the most hostile location for a tumour cell from an immunological stand point, the immune response of TDLN must be profoundly impaired. In this context, tolerance should be particularly efficient when tumour cells express foreign proteins as in cervical cancer (CC), a tumour resulting from a chronic infection with certain human papilloma virus types, usually HPV16 and HPV18 that induces the expression of viral proteins on the tumour cell surface. In line with this hypothesis, a high frequency of CD4<sup>+</sup> regulatory T cells (Treg) has been previously found in pelvic TDLN of patients with CC as compared to endometrial cancer [17] and HPV-specific Treg have been described both in mTDLN and among tumour infiltrating T cells in CC [51]. In the present study, we performed a comparative analysis of the immune system state in metastasis free TDLN (mfTDLN) and mTDLN to establish whether mTDLN have a reduced capacity to mount an efficient cell-mediated anti-tumour immune response. In addition to Treg and Treg subsets, we focused on dendritic cell (DC) subsets, as it is apparent that, although the interaction of T cells with DC is fundamental for a favourable immune response to tumours, certain DC subsets can induce tolerance rather than immunity [46]. We demonstrate that mTDLN are characterised by a significant excess of various suppressor type cells and that this tolerogenic milieu correlates with a significant Tc2 polarisation and a clear trend towards a lower content of effector type T cells. Finally, we report on the yet scarcely recognised CD8<sup>+</sup>Foxp3<sup>+</sup>T cell subset [30] and show that this cell subset is endowed with suppressive activity and tends to be more frequent in mTDLN, thereby leading one to speculate about its possible role in facilitating tumour-induced immune suppression.

## Materials and methods

### Study patients

A total of 53 consecutive patients with early stage cervical cancer (age range 34–80, median 53 years) was included in

the study. No patient underwent chemo -or chemoradiation therapy before surgery. Patients underwent radical surgery at the Gynecologic Oncology Unit, Catholic University, Rome, and at the Department of Oncology, Catholic University, Campobasso. This study was approved by the Ethical Committee of the Catholic University and written informed consent was signed by all patients. Clinico-pathological characteristics of the patients are summarised in Table 1. Staging was performed according to FIGO classification: pretreatment evaluation consisted of history and physical examination, biopsy and gynecologic examination under general anaesthesia. Surgery consisted of Type II ( $n = 23$ ) and Type III ( $n = 30$ ), radical hysterectomy according to Piver classification [38], with bilateral systematic pelvic lymphadenectomy. Most of the specimen was retained for staging, while the rest was mechanically disaggregated for immunological analysis. TDLN were defined as positive for tumour metastasis by routine histopathological evaluation and assigned to either mTDLN or mfTDLN category.

### Isolation of TDLN cells and immunostaining

Sterile mononuclear cell suspension from TDLN samples was obtained immediately after surgery, as previously

**Table 1** Clinico-pathological characteristics of patients

Clinical and pathological characteristics	Number of patients <i>n</i> (%)
FIGO stage	
IA	5 (9.4)
IB	43 (81.1)
IIA	5 (9.4)
Histotype	
Squamous	34 (64.2)
Adenocarcinoma/adenosquamous	16 (30.2)
Glossy cell	2 (3.7)
Small cell	1 (1.9)
Grade	
G1–2	25 (47.2)
G3	17 (32.0)
NA	11 (20.8)
Tumour size (cm)	
<4	49 (92.5)
≥4	4 (7.5)
Type of surgery	
Piver II	23 (43.4)
Piver III	30 (56.6)
Nodal metastasis	
Yes	11 (20.8)
No	42 (79.2)

NA Not applicable

described [4]. Four-colour flow cytometry was performed using monoclonal antibodies (mAbs) labelled with the fluorescent dyes FITC, PE, PE-Tx red, and PE-Cy5 (PC5) and appropriately combined to assess informative antigens and define cell subsets. Table 2 describes the markers that define each of the subsets of cells investigated. Optimal mAb concentrations were determined for each mAb by titration. We used mAbs to: CD19, CD3, CD4, CD8, CD62L, CD45RO, CD45RA, CD56 and HLA-DR (Beckman Coulter, Miami, FL); CD25, CD27, CD69 and CCR4 (BD Pharmingen™, San Jose, CA); chemokine receptors CCR5, CXCR3 and CCR3 (R&D System, Minneapolis, MN). mAb to neuropilin 1 (Nrp1) was BDCA4 [5] (Miltenyi Biotec, Bergish-Gladbach, Germany). It is generally admitted that, whereas myeloid DC (mDC) are immunogenic for T cells, plasmacytoid DC (pDC) may be tolerogenic and involved in maintaining peripheral immune tolerance to tumour antigens [29, 41]. pDC and mDC were assessed by the IO test® CD(14 + 16)-FITC/CD85k-PE/CD123-PC5 and CD(14 + 16)-FITC/CD85k-PE/CD33-PC5, respectively (both from Beckman Coulter), following manufacturer's instructions. Details of the procedure are reported in the data sheet, which also illustrates the gating strategy used to identify mDC and pDC. Briefly, the test takes advantage of the coordinate expression of CD33 and CD85k by CD14<sup>-</sup>CD16<sup>-</sup> cells to identify mDC and of the coordinate expression of CD123 and CD85k by CD14<sup>-</sup>CD16<sup>-</sup> cells to identify pDC [33, 36, 50]. MAb to Foxp3 was clone PCH101 (eBioscience, San Diego, CA). Foxp3 expression was determined by intracellular staining.

**Table 2** Summary of cell subsets assessed in the study

Target population	Phenotype
CD4 <sup>+</sup> T cells	
Naïve cells	CD62L <sup>+</sup> CD45RA <sup>+</sup>
Type-1 effector cells	CXCR3 <sup>+</sup> CCR5 <sup>+</sup>
Type-2 effector cells	CCR4 <sup>+</sup> CCR3 <sup>+</sup>
Regulatory cells	Foxp3 <sup>+</sup>
Activated regulatory cells	Foxp3 <sup>+</sup> CD69 <sup>+</sup>
Activated regulatory cells	Foxp3 <sup>+</sup> HLA-DR <sup>+</sup>
Functionally potent regulatory cells	Foxp3 <sup>+</sup> Nrp1 <sup>+</sup>
CD8 <sup>+</sup> T cells	
Naïve cells	CD62L <sup>+</sup> CD45RA <sup>+</sup>
Effector cells	CD45RA <sup>-</sup> CD27 <sup>+</sup>
Type-1 effector cells	CXCR3 <sup>+</sup> CCR5 <sup>+</sup>
Type-2 effector cells	CCR4 <sup>+</sup> CCR3 <sup>+</sup>
Regulatory cells	Foxp3 <sup>+</sup>
NK cells	CD3 <sup>-</sup> CD16 <sup>+</sup> CD56 <sup>+</sup>
Myeloid dendritic cells	CD14 <sup>-</sup> CD16 <sup>-</sup> CD85k <sup>+</sup> CD33 <sup>+</sup>
Plasmacytoid dendritic cells	CD14 <sup>-</sup> CD16 <sup>-</sup> CD85k <sup>+</sup> CD123 <sup>+</sup>

To this end, cells were stained for surface antigens, washed and then fixed and permeabilized using the staining kit provided by the manufacturer. With permeabilized lymphocytes, mAb can give increased background fluorescence, possibly due to entry of free fluorochrome and/or mAb reactivity with charged or polar internal molecules that cannot be established correctly by the conventional isotype staining. As outlined earlier [3], we overcame this complication incubating permeabilized cells first with a tenfold molar excess of unlabelled anti-Foxp3 mAb PCH101 clone (eBioscience) 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 5,000 cells of interest were acquired for each sample. List mode data were analysed using Expo 32™ (Beckman Coulter) software.

#### Functional assay

All cell populations to be used in functional assays were obtained by immunomagnetic cell sorting (Miltenyi Biotec), according to manufacturer's instructions. Cell surface CD25 was used as a marker to sort Treg from CD4<sup>+</sup> cells for functional studies. Thus, the population tested necessarily contained a mix of regulatory, i.e., CD25<sup>int/high</sup>Foxp3<sup>+</sup> and nonregulatory, CD25<sup>int/high</sup>Foxp3<sup>-</sup> T cells. Similar considerations apply to CD8<sup>+</sup> T cells. However, in functional studies these purified populations will be operationally defined as Treg and CD8<sup>+</sup>Treg, respectively. To purify Treg and CD8<sup>+</sup>Treg, cell suspensions were at first enriched in either CD4<sup>+</sup> or CD8<sup>+</sup> cells by double positive selection using CD4 and CD8 multisort kit and MS columns. Afterwards, a double positive selection was performed using CD25 microbeads and MS columns. This procedure routinely produced an enrichment in CD25<sup>high</sup> cells (usually ≥25% of all sorted CD25<sup>+</sup> population). Various numbers of responder and regulatory cells were seeded in replicate wells in a standard flat-bottomed 96-well culture plate (Falcon) precoated overnight with a mixture of anti-CD3 (clone UCHT-1) and anti-CD28 (clone YTH913.12 1 µg/ml, Serotec Ltd, Oxford, UK). PHA (1.5 µg/ml, Sigma San Louis, MO) was also used and gave results essentially analogous to CD3/CD28 stimulation. Responder cells were allogenic peripheral blood mononuclear cells (PBMC) immunomagnetically depleted of CD25 expressing cells. Incubation was carried out at 37°C in a 5% CO<sub>2</sub> atmosphere for 5 days. The response of T cells to polyclonal activation was assessed using carboxyfluorescein diacetate succinimidyl ester (CFSE, Molecular Probes, Eugene, OR). The staining procedure was as described [4] and the number of cell divisions quantified by ModFit™/Cell

Proliferation Model™ software (Sigma San Louis, MO). In a previous study [4], we reported that Treg in TDLN suppressed both CD4<sup>+</sup> and CD8<sup>+</sup> T cells. Thus, experiments were also designed to assess separately the regulatory capacity of CD8<sup>+</sup>Treg on CD4<sup>+</sup> and CD8<sup>+</sup> T cells. For this end, we used the whole cell population of CD25-depleted PBMC as responder cells and the intracellular covalent coupling dye CFSE [4]. At the end of culture, cells were stained with PE-CD8 mAb to quantify the number of cell divisions in relationship with the expression of the membrane marker. Since the vast majority of CD8<sup>-</sup> cells after 4 days of culture of PBMC are T cells, CD8<sup>-</sup> subset response was considered equivalent to that of the CD4<sup>+</sup> subset. This approach was preferred over the direct identification of the CD4<sup>+</sup> subset by anti-CD4 mAb because it has been demonstrated previously that the surface CD4 molecule is downmodulated in activated lymphocytes [42], whereas CD8 expression does not diminish. The suppressive activity of Nrp1<sup>+</sup>Treg was tested [3]. Briefly, T cells were purified by a double positive selection using the PE-CD3 mAb, anti-PE multisort kit, and LS columns. Next, CD25<sup>int/high</sup> cells were obtained by double positive selection using FITC-CD25 mAb and anti-FITC multisort kit, and MS columns. Nrp1<sup>+</sup> cells were finally purified by double positive selection using BDCA4 microbeads and MS columns. These cells will be thereafter referred to as Nrp1<sup>+</sup>Treg. The final purity of Nrp1<sup>+</sup>Treg population used in functional assays was at least 60%.

#### Immunohistochemistry

Immunostaining was performed on 3 μ paraffin tissue section mounted on poly-l-lysine-coated slides [18]. Briefly, slides were deparaffinized, rehydrated, and the endogenous peroxidase blocked with 3% H<sub>2</sub>O<sub>2</sub>. Antigen retrieval procedure was performed by microwave oven heating in 10 mM citric acid, pH 6.0. Non-specific binding was reduced using 20% normal goat serum. VEGF and Nrp1 expressing cells were identified after overnight incubation at 4°C by using a polyclonal rabbit anti-VEGF antibody (Santa Cruz Technology) and an anti-Nrp1 mouse mAb clone A-12 (Santa Cruz Biotechnology, Santa Cruz, CA), respectively. Reagents were used at 1:50 dilution. Anti-VEGF antibody binding was evaluated by the EnVision-rabbit + System-HRP (Dako, Carpinteria, CA) and diaminobenzidine as a chromogen (DAB substrate System, Dako), whereas anti-Nrp1 mAb binding was evaluated by the EnVision System AP (Dako) and Fast-Red as a chromogen (Fast-Red substrate pack, ScyTek Laboratories, UT), according to manufacturer's instructions. Sections were counterstained with haematoxylin and mounted with Eukitt. Negative control was obtained by omission of the primary antibody.

#### Statistical analysis

Immune profile of TDLN in relationship with the presence of infiltrating tumour cells was analysed using Student's *t* test.

#### Results

##### Immune profile of TDLN in relationship with the presence of metastatic tumour cells

As a first approach to understand whether the presence of infiltrating tumour cells affected the immune profile of TDLN, we assessed the frequency of the main lymphoid cell populations commonly colonising lymph nodes, i.e., T and B cells, CD4<sup>+</sup> and CD8<sup>+</sup> T cells, and NK cells. As shown in Table 3, mTDLN contained significantly more B cells and less T cells than mfTDLN. The decrease in T cells involved both CD4<sup>+</sup> and CD8<sup>+</sup> cells (Table 3). mTDLN and mfTDLN contained similar small amounts of NK cells (Table 3). Steps required for an effective anti-tumour response include capture of tumour antigens by DC and presentation to T cells followed by activation and expansion of antigen-specific T cells. However, DC can be actively immunosuppressive and do not activate T cells but rather induce tolerance to tumour [46]. Thus, we extended the analysis to changes involving DC. When the frequency of total DC as well as pDC and mDC populations was analysed between mTDLN and mfTDLN, we did not observe significant differences, although pDC frequency slightly increased and mDC slightly decreased in mTDLN (Table 3). Even though these differences were tiny, they were suggestive for an imbalance between tolerogenic, i.e., plasmacytoid and immunogenic, i.e., myeloid DC.

**Table 3** Percentages of immune cell populations in mTDLN and mfTDLN

	mTDLN (n = 8)	mfTDLN (n = 41)	P value
B cells	44 ± 5 <sup>a</sup>	34 ± 8	0.020
T cells	50 ± 13	60 ± 8	0.012
CD4 <sup>+</sup> T cells	44 ± 11	50 ± 8	0.071
CD8 <sup>+</sup> T cells	9 ± 4	10 ± 3	0.114
NK cells	2.2 ± 1.8	2.1 ± 1.1	0.821
DC	1.0 ± 0.3	1.1 ± 0.8	0.75
pDC	0.82 ± 0.23	0.76 ± 0.50	0.957
mDC	0.17 ± 0.19	0.28 ± 0.40	0.598
pDC/mDC ratio	9 ± 7	4 ± 3	0.022

*mTDLN* Metastatic tumour draining lymph nodes, *mfTDLN* metastasis free tumour draining lymph nodes, *DC* dendritic cells, *pDC* plasmacytoid DC, *mDC* myeloid DC

<sup>a</sup> Data are expressed as mean ± SD

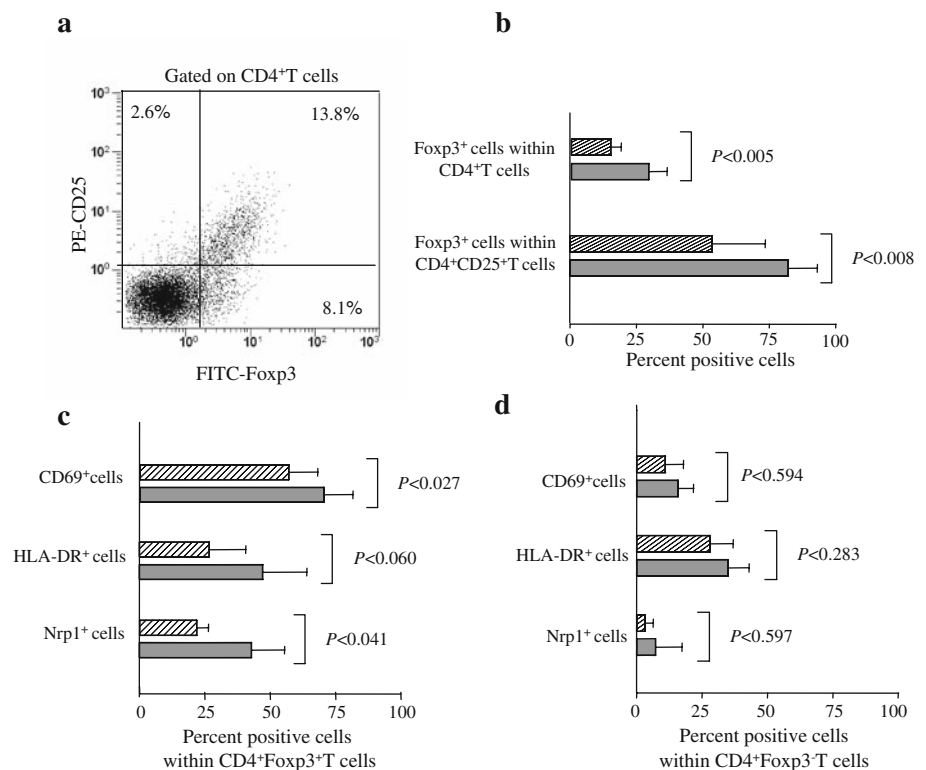
Thus, we calculated the pDC/mDC ratio for each mfTDLN and mTDLN and expressed this data as mean  $\pm$  SD (Table 3). Student's *t* test was then applied and showed that the pDC/mDC ratio was significantly higher in mTDLN (Table 3).

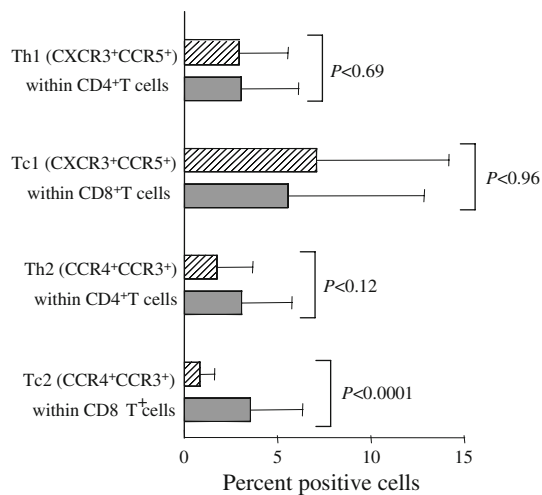
We next moved to investigate the frequency and activation state of Treg. Treg were originally defined as CD4<sup>+</sup>T cells preferentially expressing high levels of CD25 (CD25<sup>high</sup>) [43]. More recently, the nuclear expression of the transcription factor Foxp3, which is required for the development and function of Treg, has been indicated as best option for Treg detection to date [54]. Since all available information on Foxp3 and CD25 expression in human CD4<sup>+</sup>T cells is from peripheral blood studies, we first established the relationship between CD25 and Foxp3 in CD4<sup>+</sup>T cells in TDLN. Figure 1a shows that Foxp3 and CD25 expression levels were directly related. However, a sizeable amount of Foxp3<sup>+</sup> cells lacked CD25 expression and a small amount of CD25<sup>int</sup> cells was Foxp3<sup>-</sup>. This pattern of expression of CD25 and Foxp3 was uniformly observed in mfTDLN and mTDLN and is quite similar to the circulating Treg [32, 54], thereby indicating comparable CD25 and Foxp3 expression modalities in the two compartments. Treg, expressed as CD4<sup>+</sup>Foxp3<sup>+</sup> cell frequency within the CD4<sup>+</sup>T cell population, were significantly more abundant in mTDLN than mfTDLN (Fig. 1b). To take into account, CD25 expression according to an earlier study [32], we evaluated Foxp3 expressing cells within CD4<sup>+</sup>CD25<sup>+</sup> T cells and found that also these cells were

significantly more abundant in mTDLN (Fig. 1b). Next, we moved to analyse the activation state of Treg as a further indicator of tolerogenicity and found that Treg in mTDLN were more activated than in mfTDLN, as indicated by the significant increase in CD69 and the enhanced, although not statistically significant, HLA-DR expression [20, 47] (Fig. 1c). Following the seminal observation that murine Treg may express Nrp1 [7], we showed earlier that Nrp1<sup>+</sup>Treg also exist in humans and are more efficient than their Nrp1<sup>-</sup> counterpart in inhibiting T cell responsiveness [3]. Thus, we compared the frequency Nrp1<sup>+</sup>Treg in mTDLN to that in mfTDLN and found that Nrp1<sup>+</sup>Treg were significantly more abundant in mTDLN (Fig. 1c). As a comparison, we searched for the expression of activation markers and Nrp1 by non-Treg, i.e., CD4<sup>+</sup>Foxp3<sup>-</sup> T cells. As shown in Fig. 1d, the percentages of positive cells in mTDLN and mfTDLN did not diverge significantly.

We next moved to investigate changes in T cell subsets generally regarded as indicators of anti-tumour response within each of the major T cell subset, i.e., CD4<sup>+</sup> and CD8<sup>+</sup> cells. CD4<sup>+</sup>T helper (Th) cells and CD8<sup>+</sup>T cytotoxic (Tc) cells can be polarised to Th1 and Tc1 cells, implicated in cellular immunity, and Th2 and Tc2 cells, implicated in humoral immunity and generally of less importance in tumour immunology [9, 15]. Here we used the coexpression of CCR5 and CXCR3 on CD4<sup>+</sup> and CD8<sup>+</sup> T cells as an indicator for Th1 and Tc1 cells, respectively, and the coexpression of CCR3 and CCR4 on CD4<sup>+</sup> and CD8<sup>+</sup> T

**Fig. 1** Treg assessment in mfTDLN and mTDLN. **a** Cells of a representative mTDLN were mechanically dissociated and analysed by flow cytometry to assess CD25 and Foxp3 expression modality on CD4<sup>+</sup>T cells. Numbers in quadrants indicate the percentage of cells expressing the relevant marker. **b** Percentage of Foxp3<sup>+</sup> cells within CD4<sup>+</sup>T cells and percentage of Foxp3<sup>+</sup> cells within CD4<sup>+</sup>CD25<sup>+</sup>T cells. Histograms show mean values  $\pm$  SD from analyses performed in 31 mfTDLN (hatched bars) and 7 mTDLN (filled bars). **c** Percentage of CD69<sup>+</sup>, HLA-DR<sup>+</sup> and Nrp1<sup>+</sup> cells within CD4<sup>+</sup>Foxp3<sup>+</sup>T cells and **d** within CD4<sup>+</sup>Foxp3<sup>-</sup>T cells. Histograms show mean values  $\pm$  SD from analyses performed in 28 mfTDLN (hatched bars) and 6 mTDLN (filled bars)





**Fig. 2** Type 1 and type 2 polarisation in mTDLN and mTDLN. Th1 and Tc1 cells were assessed by staining T cells with CXCR3, CCR5 and either CD4 (Th1) or CD8 (Tc1). Th2 and Tc2 cells were assessed by staining T cells with CCR4, CCR3 and either CD4 (Th2) or CD8 (Tc2). Percentages of cells positive for the various markers within CD4<sup>+</sup> and CD8<sup>+</sup> T cells in mTDLN (hatched bars) or mTDLN (filled bars). Histograms show mean values  $\pm$  SD from analyses performed in 35 mTDLN and 7 mTDLN

cells as an indicator for Th2 and Tc2 cells, respectively [24, 40, 44]. Th1 and Tc1 cell frequency was comparable in mTDLN and mTDLN (Fig. 2), whereas Tc2 cells showed a significant association with the presence of tumour cells and, consistently, Th2 cells were substantially increased in mTDLN (Fig. 2). A clear trend towards a lower presence of effector type CD8<sup>+</sup>CD27<sup>-</sup>CD45RA<sup>+</sup> cells [1] in mTDLN ( $2.3 \pm 0.5$  vs.  $11.8 \pm 22.2$ , mTDLN and mTDLN, respectively), that, however, did not achieve statistical significance ( $P < 0.39$ ) could be discerned.

There was no significant difference in the frequency of naïve CD4<sup>+</sup>CD45RA<sup>+</sup>CD45RO<sup>-</sup>CD62L<sup>+</sup> and CD8<sup>+</sup>CD45RA<sup>+</sup>CD45RO<sup>-</sup>CD62L<sup>+</sup> T cells [13] (naïve CD4<sup>+</sup> T cells  $21 \pm 17$  vs.  $24 \pm 15$ , mTDLN and mTDLN, respectively; naïve CD8<sup>+</sup> T cells  $20 \pm 21$  vs.  $26 \pm 22$ , mTDLN and mTDLN, respectively).

#### Functional studies on Treg in mTDLN and mTDLN

The phenotypic profile of Treg in mTDLN was suggestive for an enhanced suppressor activity. In an attempt to verify this hypothesis, Treg were immunomagnetically sorted from mTDLN and mTDLN and analysed for their ability to block responsiveness to mitogenic stimulation of T cells in vitro. Figure 3a shows that CD69, HLA-DR and Nrp1 expression in Treg immunomagnetically purified from mTDLN was non-significantly higher than in Treg from mTDLN. Treg from mTDLN and mTDLN inhibited T cell proliferation with a comparable efficiency (Fig. 3b). As outlined above, Nrp1 defines a potent Treg subset in human

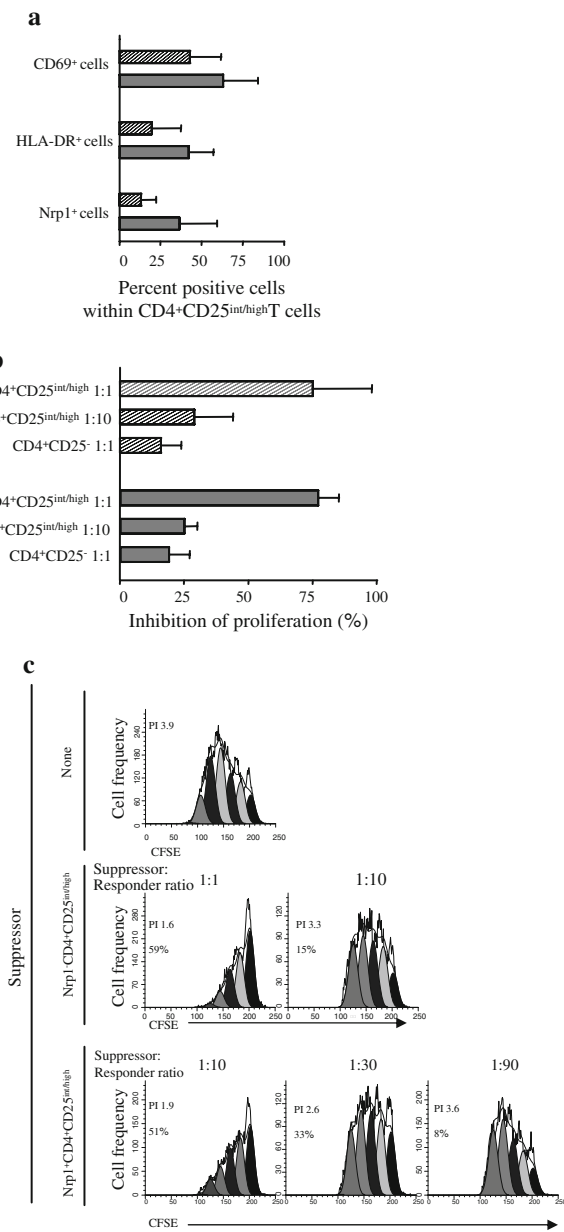
**Fig. 3** Suppression of T cell proliferation by CD4<sup>+</sup>CD25<sup>int/high</sup> T cells in mTDLN and mTDLN, and superior suppressive activity of Nrp1<sup>+</sup>CD4<sup>+</sup>CD25<sup>int/high</sup> T cells in mTDLN. **a** Percentage of CD69, HLA-DR and Nrp1 expressing cells within immunomagnetically purified CD4<sup>+</sup>CD25<sup>int/high</sup> T cells. Histograms show mean values  $\pm$  SD from analyses performed in 3 mTDLN (hatched bars) and 2 mTDLN (filled bars). **b** Immunomagnetically purified CD4<sup>+</sup>CD25<sup>int/high</sup> T cells and CD4<sup>+</sup>CD25<sup>-</sup> T cells as a control were cultured with CFSE-loaded CD25-depleted allogenic responder PBMC ( $5 \times 10^4$ /well). Suppressor activity was tested at the indicated suppressor:responder ratios. The proliferative response was assessed on day 5 by computing the proliferation index by ModFit™/Cell Proliferation Model™ software and suppressive activity expressed as percent inhibition. Histograms show mean values  $\pm$  SD from experiments carried out in 3 mTDLN (hatched bars) and 2 mTDLN (filled bars). **c** Nrp1<sup>+</sup>CD4<sup>+</sup>CD25<sup>int/high</sup> T cells and Nrp1<sup>-</sup>CD4<sup>+</sup>CD25<sup>int/high</sup> T cells from mTDLN were immunomagnetically sorted from CD4<sup>+</sup>CD25<sup>int/high</sup> T cells and cultured with CFSE-loaded CD25-depleted allogenic responder PBMC ( $5 \times 10^4$ /well) at the indicated suppressor:responder ratios. At day 5 of culture, proliferation index (PI) was computed by ModFit™/Cell Proliferation Model™ software. PI and percent inhibition in each culture condition are shown. Data of one of two experiments with similar results are shown

mTDLN [3]. Thus, we also tested whether Nrp1<sup>+</sup>Treg in mTDLN were more potent than their Nrp1<sup>-</sup> counterpart in antagonizing the proliferative response of T cells. Data in Fig. 3c show that the suppressor activity of Nrp1<sup>-</sup>Treg became marginal at a suppressor/responder ratio of 1:10 while that of Nrp1<sup>+</sup>Treg became marginal at a suppressor/responder ratio of 1:90, consistent with a superior suppressive capacity.

#### CD8<sup>+</sup>Treg in mTDLN and mTDLN

Some lines of evidence indicate that certain CD8<sup>+</sup>T cells may express CD25 and/or Foxp3 and have suppressor capability [49]. Thus, we first assessed Foxp3 expression by CD8<sup>+</sup>T cells in relationship with CD25 expression. Figure 4a exemplifies the expression modality of Foxp3 and CD25 in CD8<sup>+</sup>T cells in a mTDLN. Sizeable proportions of CD8<sup>+</sup>T cells expressed Foxp3, CD25 or both (Fig. 4a). As in Treg, we measured CD8<sup>+</sup>Treg as the frequency of Foxp3<sup>+</sup> cells within the CD8<sup>+</sup>T cell population and measured Foxp3 expressing cells within CD8<sup>+</sup>CD25<sup>+</sup> T cells. Both cell subsets were more abundant in mTDLN than mTDLN, although the differences were not significant (Fig. 4b).

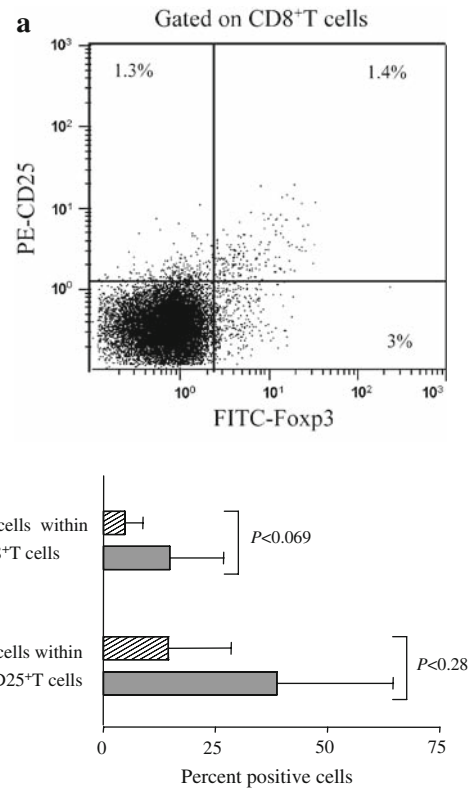
To provide direct evidence that CD8<sup>+</sup>Treg were actual suppressor cells, CD8<sup>+</sup>Treg were immunomagnetically sorted from mTDLN and analysed for their ability to block mitogenic stimulation of T cells in vitro. Adding suppressor cells into cultures with a fixed dose of responder cells led to a marked decrease in proliferation of the latter (Fig. 5a). Both CD8<sup>+</sup>Treg and Treg mediated inhibition was evident at a suppressor/responder ratio of 1:1 and marginal at a suppressor/responder ratio of 1:10 (Fig. 5a), suggesting a similar suppressive potency by the two subsets. Treg



abrogate the proliferation of both CD4<sup>+</sup> and CD8<sup>+</sup> T cells [4]. To test whether this applied to CD8<sup>+</sup>Treg too, immunomagnetically sorted CD8<sup>+</sup>Treg were cocultured with responder cells for 5 days in the presence of PHA as polyclonal stimulus. CD8<sup>+</sup> responder cells were directly identified by PE-CD8 staining, whereas CD4<sup>+</sup> responder cells were indirectly identified as CD8<sup>-</sup> cells. As shown in Fig. 5b, CD8<sup>+</sup>Treg efficiently inhibited proliferation of both CD4<sup>+</sup> and CD8<sup>+</sup> responder cells.

**Immunostaining for Nrp1 and VEGF in mTDLN**

We were interested in assessing whether metastatic cancer cells produced VEGF, because this cytokine is one of the

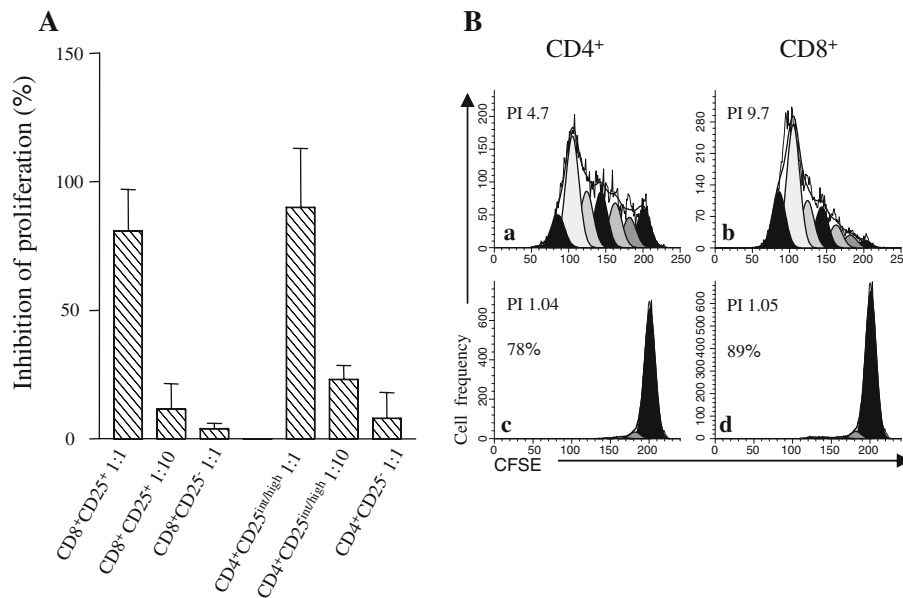


**Fig. 4** CD8<sup>+</sup>Foxp3<sup>+</sup>T cells assessment in mTDLN and mTDLN. **a** Cells of a representative mTDLN were mechanically dissociated and analysed by flow cytometry to assess CD25 and Foxp3 expression modality on CD8<sup>+</sup>T cells. *Numbers* in quadrants indicate the percentage of cells expressing the relevant marker. **b** Percentage of Foxp3<sup>+</sup> cells within CD8<sup>+</sup>T cells and percentage of Foxp3<sup>+</sup> cells within CD8<sup>+</sup>CD25<sup>+</sup>T cells. Histograms show mean values ± SD from analyses performed in 26 mTDLN (*hatched bars*) and 6 mTDLN (*filled bars*)

natural ligands for Nrp1 [21] present on the cell surface of Nrp1<sup>+</sup>Treg and pDC [5, 6, 35] and may therefore provide a mechanistic insight into the interaction between tumour-derived factors and immune cells. Immunohistochemistry showed that most metastatic tumour cells produced VEGF (Fig. 6, left panel). We also attempted to establish a possible spatial relationship between Nrp1<sup>+</sup>Treg, pDC and VEGF expressing tumour cells. pDC were found interspersed among VEGF<sup>+</sup> metastatic tumour cells (Fig. 6, right panel). Disappointedly, the anti-Nrp1 mAb failed to reliably stain lymphoid cells, possibly reflecting the low level of Nrp1 expression in Treg as compared to pDC (more than 1 log difference by flow cytometry [3]).

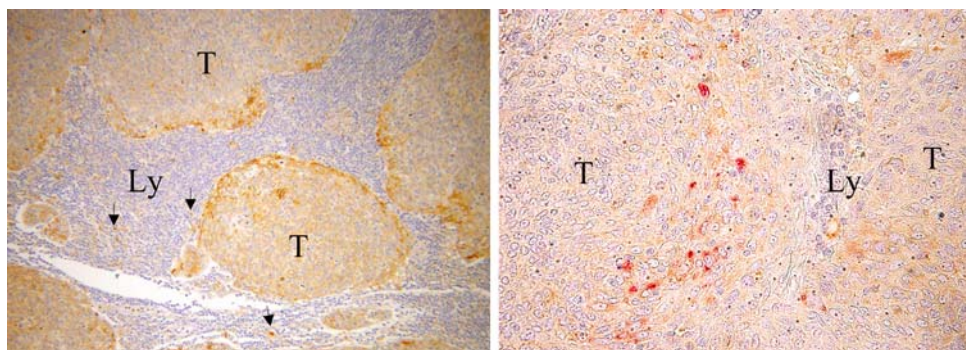
**Discussion**

Both mTDLN and mTDLN are under the influence of tolerogenic factors imported from the tumour area, that turn them into a site in which immune suppression rather



**Fig. 5** Suppression of T cell proliferation by CD8<sup>+</sup>Foxp3<sup>+</sup>T cells. **A** Immunomagnetically purified CD8<sup>+</sup>CD25<sup>+</sup>T cells and, as comparison, CD4<sup>+</sup>CD25<sup>int/high</sup>T cells were incubated with CFSE-loaded CD25-depleted allogenic responder PBMC ( $5 \times 10^4$ /well). Controls included CD8<sup>+</sup>CD25<sup>-</sup> and CD4<sup>+</sup>CD25<sup>-</sup> T cells. Suppressor activity was tested at the indicated suppressor:responder ratios. The proliferative response was assessed on day 5 by computing the proliferation index by ModFit™/Cell Proliferation Model™ software and suppressive activity expressed as percent inhibition. Histograms show mean values  $\pm$  SD from experiments carried out in 3 mTDLN. **B** The antiproliferative

capacity of CD8<sup>+</sup>CD25<sup>+</sup>T cells on allogenic CD4<sup>+</sup> and CD8<sup>+</sup> T cells was tested by culturing CFSE-loaded CD25-depleted allogenic responder PBMC ( $5 \times 10^4$ /well) in the absence (**a, b**) or in the presence (**c, d**) of CD8<sup>+</sup>CD25<sup>+</sup>T cells at a suppressor:responder ratio of 1:1. At day 5 of culture, responder cells were stained with PE-CD8 mAb, CFSE fluorescence histograms obtained separately for CD4<sup>+</sup>, i.e., CD8<sup>-</sup>, (a, c), and CD8<sup>+</sup> (b and d) cells, and PI calculated by ModFit™/Cell Proliferation Model™ software. PI and percent inhibition in each culture condition are shown. Representative of one of two separate experiments carried out in mTDLN



**Fig. 6** Immunostaining for VEGF and Nrp1 in mTDLN. *Left panel* metastatic tumour cells (*T*) showing accumulation of VEGF immunoreaction in the cytoplasm. VEGF was mostly expressed by tumour cells facing lymphoid tissue (*Ly*). Scattered stromal cells, likely belonging to the macrophage lineage [2], are also VEGF<sup>+</sup> (*arrows*). Original

magnification  $\times 100$ . *Right panel* Nrp1<sup>+</sup>pDC (*reddish* in the online publication) are visible among VEGF<sup>+</sup> tumour cells (*brownish* in the online publication). VEGF staining in right panel was intentionally maintained low by reducing incubation time and mAb concentration so as not to interfere with Nrp1 staining. Original magnification  $\times 400$

than immune response originates [10, 34, 55]. Our work was designed to determine whether mTDLN, being also exposed to tolerogenic factors locally produced by the infiltrating tumour cells, were more immunosuppressed than mTDLN. We first analysed the relative proportions of the main lymphocyte populations and found a significant excess of B cells at the expense of T cells in mTDLN. A similar alteration was reported earlier in mTDLN of

breast cancer [31] and melanoma patients [16], suggesting that such an imbalance between B and T cells represents a generalised pattern of mTDLN, irrespective of tumour type. The processes underlying the relative T cell loss are obscure. It may be hypothesised that tumour cells prefer to invade the T cell areas. However, a comparison of present data with the available literature data on B and T cell distribution in normal human lymph nodes [8, 52], indicates that



a relative T cell loss takes place also in mTDLN. This observation argues that such an altered B to T cell ratio is a common feature of TDLN.

When we examined mTDLN and mfTDLN for their content of immune cell populations representative of tolerance, we found that Treg were significantly more frequent in mTDLN. This finding is reminiscent of Treg accumulation documented in certain primary tumour sites [12]. Thus, it is plausible that metastatic tumour cells tend to recreate in mTDLN a tolerogenic milieu to protect them against immune attack. The overrepresentation of Treg in mTDLN as compared to mfTDLN is not confined to CC. Viguiet et al. [53] and Jandus et al. [25] showed the Treg were a major component of the immunosuppressive microenvironment of mTDLN in melanoma. The finding that Treg in mTDLN were more activated than in mfTDLN and enriched in Nrp1 expressing cells indicates that in addition to favouring Treg trafficking into mTDLN, metastatic tumour cells privileged the accumulation of Treg subsets particularly efficient in sabotaging local immune response [3]. In regard to this, being VEGF one of the natural ligands for Nrp1 [21], it is tempting to speculate that VEGF produced by metastatic cancer cells may facilitate the suppressive activity of Nrp1<sup>+</sup>Treg, in line with the known suppressor capability of this cytokine in the context of tumour immunology [35]. Unfortunately, attempts to formally prove or disprove the supposed superior immunosuppressive capacity of Treg in mTDLN have been thwarted by the scarcity of mTDLN yielding enough viable Treg to be tested functionally.

Both mfTDLN and mTDLN contained substantial amounts of CD8<sup>+</sup>Treg, as identified on the basis of the constitutive expression of Foxp3. Data on suppressor CD8<sup>+</sup>Foxp3<sup>+</sup> T cells are scanty. Indeed, CD8<sup>+</sup> T cells constitutively expressing Foxp3<sup>+</sup> were first found among CD8<sup>+</sup>CD25<sup>+</sup> single positive thymocytes [11]. More recently, passing reference has been made to the presence of a minute amount of circulating CD8<sup>+</sup> cells constitutively expressing Foxp3 in healthy individuals and prostate cancer patients [30]. Peripheral CD8<sup>+</sup> T cells endowed with a regulatory ability have been identified in different clinical settings [27, 39] but none expressed Foxp3 unless activated in vitro. It has been proposed that these cells can be recruited to the sites of active immune responses as pre-existing circulating Foxp3<sup>-</sup> regulatory cells [26]. This would explain why CD8<sup>+</sup>Foxp3<sup>+</sup> cells are exceedingly rare in peripheral blood but, as we have shown here, are substantially enriched in TDLN. In an initial characterisation of CD8<sup>+</sup>Treg functionality, we tested their ability to prevent T cell proliferation and found that, as in Treg, CD8<sup>+</sup>Treg efficiently inhibited both CD4<sup>+</sup> and CD8<sup>+</sup> T cell responsiveness to polyclonal activation in vitro. Ongoing studies will clarify whether CD8<sup>+</sup>Treg and Treg in TDLN share the

same mechanisms of suppression, for example, whether suppression is contact- or cytokine-dependent. In regard to this, recent evidence indicates that CD8<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T cells can be derived from prostate tumour tissue and share some suppressive mechanisms with Treg [30]. Although preliminary, present data leads one to speculate that CD8<sup>+</sup>Treg are suppressor cells and their enhanced presence in mTDLN, similar to Treg, may imply that the two suppressor T cell subsets act in concert to induce a tolerogenic milieu.

mTDLN were characterised by a significantly increased pDC/mDC ratio. It is generally admitted that, whereas mDC are immunogenic for T cells, pDC may be tolerogenic and even promote Treg development [41, 48]. Thus, the relative excess of pDC appears consistent with the tolerogenic profile of mTDLN and the enhanced presence of Treg. Immunohistochemical analysis showed that pDC were interspersed among VEGF<sup>+</sup> metastatic tumour cells. pDC constitutively express Nrp1 [5]. Thus, as VEGF is one of the natural ligands for Nrp1 [21], this finding may be indicative of a mechanism by which metastatic tumour cells interact with pDC, possibly promoting their tolerogenic activity. An in vitro study demonstrated that Nrp1 can be passively acquired by T cells following cell-to-cell contact with Nrp1<sup>+</sup> antigen presenting cells [6]. Thus, the relative pDC excess may also offers a mechanism explaining the higher amount of Nrp1<sup>+</sup>Treg in mTDLN.

The importance of the increased frequency of suppressor cells in limiting immunological reactivity in mTDLN was underscored by the significant Tc2 polarisation and substantially reduced presence of effector type CD45RA<sup>+</sup>CD27<sup>-</sup>CD8<sup>+</sup> T cells. In regard to this, studies in mice demonstrated that Treg directly hamper the generation and accumulation of cytotoxic T cells in tumour microenvironment [14] and a concomitant increase of Treg and reduction of effector T cells has been described in primary tumour site in CC [37].

Several lines of evidence indicate that, while primary tumour creates the tolerogenic milieu in TDLN, the latter contributes to exert a tolerizing effect on the whole immune system, mainly by exporting activated, tumour specific suppressor cells [34]. By showing that metastatic invasion of TDLN forced the generation of a tolerogenic milieu even further, present data suggest that mTDLN are even more efficient in inducing systemic immune suppression. CC is currently investigated as a possible candidate for immunotherapeutic strategies [28, 45]. Thus, the results from the present study may have implications for the efficacy of vaccination therapies, obviously dependent on a functionally intact immune system. Concurrent chemoradiation has been widely recognised as the golden standard for the management of bulky stage IB and locally advanced CC [22]. However, radical surgery after chemoradiation has been

also explored [23] to remove chemoresistant foci, including mTDLN [19]. In the context of immunotherapeutic protocols, mTDLN removal would not just eliminate a tumour cell reservoir but also a source of immune suppressive cells.

Most conclusions presented here are based on flow cytometry data. However, flow cytometry does not inform about changes in absolute numbers of the analysed cell populations. Moreover, in cells colonising a tissue, it is quite difficult to integrate the total number of extracted cells into the organ of origin, especially when only fragments of tissue are available for research purposes, most of the material being retained for staging. However, in one study the absolute number of CD4<sup>+</sup>CD25<sup>+</sup>T cells in several human lymphoid tissues was shown to be equivalent to percentage [12].

In conclusion, present findings show that metastatic tumour cells confer to TDLN immunosuppressive features and alter the balance between antitumour and protumour immunity. The extent to which the tolerogenic milieu in mTDLN can induce generalised immunological alterations remains to be studied. However, as lymphocytes are mobile cells continuously recirculating between the blood and the tissues via the lymphatic system, the immune competence of TDLN is arguably reflected in the periphery. Thus, the mTDLN has to be regarded as an important contributor to the generalised immune suppression state occurring in tumour patients. Its enhanced tolerogenic capacity may pose major obstacles to effective immunotherapy.

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