Foxp3 Expressing CD4$^+$CD25^{high} Regulatory T Cells Are Overrepresented in Human Metastatic Melanoma Lymph Nodes and Inhibit the Function of Infiltrating T Cells[1]

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Dominant tolerance is mediated by regulatory T cells (T_{reg}) that control harmful autoimmune T cells in the periphery. In this study, we investigate the implication of T_{reg} in modulating infiltrating T lymphocytes in human metastatic melanoma. We found that CD4$^+$CD25^{high} T cells are overrepresented in metastatic lymph nodes (LNs) with a 2-fold increased frequency compared with both tumor-free LNs and autologous PBMCs. These cells express the Foxp3 transcription factor, display an activated phenotype, and display a polyclonal TCR Vβ chain repertoire. They inhibit in vitro the proliferation and cytokine production of infiltrating CD4$^+$CD25$^-$ and CD8$^+$ T cells (IL-2, IFN-γ) through a cell-contact-dependent mechanism, thus behaving as T_{reg}. In some cases, the presence of T_{reg} type 1/Th3-like lymphocytes could also be demonstrated. Thus, T_{reg} are a major component of the immunosuppressive microenvironment of metastatic melanoma LNs. This could explain the poor clinical response of cancer patients under immunotherapeutic protocols, and provides a new basis for future immunotherapeutic strategies counteracting in vivo T_{reg} to reinforce local antitumor immune responses. ***The Journal of Immunology*****, 2004, 173: 1444–1453.**

Melanoma is considered as an important immunogenic tumor model because of the well-known occurrence of spontaneous regressions (1), the identification of numerous tumor-associated Ags (2), as well as the detection of Ag-experienced antitumor-specific T cells in vivo (3). Attempts to induce specific antitumoral T cell responses through various immunotherapeutic protocols have revealed the potential of vaccination in inducing in some cases regression of large tumor burden. However, such favorable outcomes are still limited and await further analysis of correlations between antitumor T cell responses and clinical improvement (4–9). The reasons behind the limited success of these approaches are still largely unknown. It is possible that the induced T cell responses are not the most appropriate in terms of quantity, differentiation, or capacity to home tumor sites. In contrast, the tumor itself may have developed immune escape strategies, including impairment of Ag presentation or secretion of immunosuppressive molecules like IL-10 or TGF-β (10). However, the in vivo nature of such a T cell counteractive microenvironment remains largely unknown.

The recent description of thymic-derived CD4$^+$CD25^{high} regulatory T cells (T_{reg})[3] (3) in rodents revealed their critical role in immune tolerance and the control of autoimmunity. These T_{reg} are able to inhibit harmful autoimmune T cells in a contact-dependent and cytokine-independent mechanism (11–14). Consequently, these cells might also impair antitumor immune responses that are known to be directed at least partly against autoantigens expressed by the tumor cells (2, 3). Indeed, recent evidence for such a role has been reported in transplantable murine tumor models, where tumor rejection was increased upon elimination of CD4$^+$CD25^{high} T cells (15–17). This population of T_{reg} have been shown recently to express Foxp3, a forkhead/winged helix transcription factor that is disrupted in Scurfy mouse and in the human immune dysregulation polyendocrinopathy enteropathy X-linked syndrome, both lacking CD4$^+$CD25^{high} T_{reg}. Foxp3 appears critical for the development and function of T_{reg} (18–21). Besides Foxp3-expressing T_{reg}, other subsets of T_{reg}, such as T_{reg} type 1 (Tr1) or Th3 cells, could be generated under specific induction protocols and possibly during the course of immune responses. These subsets of T_{reg} appear to exert their suppressive activity in a contact-independent and cytokine-dependent mechanism (22, 23). It is not yet known whether these cells express the Foxp3 transcription factor. In humans, Foxp3 expressing CD4$^+$CD25^{high} T cells similar to the rodent thymic-derived CD4$^+$CD25^{high} T_{reg} have been recently described among PBMCs and thymocytes (24–33). The involvement of such CD4$^+$CD25^{high} T_{reg} in cancer patients was recently questioned by different groups. Some of them studied T_{reg} from PBMCs of colorectal cancer or melanoma patients (34–36), while others suggested the presence of these cells among tumor-infiltrating lymphocytes in breast, ovarian, and lung cancer, and more recently in Hodgkin lymphoma (37–40). However, the possible implication of CD4$^+$CD25^{high} T_{reg} in down-regulating antitumor

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[3] Abbreviations used in this paper: T_{reg}, regulatory T cells; Tr1, T_{reg} type 1; LN, lymph node; GITR, glucocorticoid-induced TNFR; HPRT, human hypoxanthine ribosyltransferase 1; MFI, mean fluorescence intensity; CDR3, complementarity-determining regions 3; int, intermediate; TCRBV, TCR Vβ.

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responses, which could explain the poor clinical response of cancer patients under immunotherapeutic protocols, remains to be demonstrated.

In the present study, we investigated the idea that T_{reg} could be involved in the control of the local immune response in human metastatic melanoma. We found that the frequency of $CD4^+CD25^{high}$ T cells is clearly increased in metastatic lymph nodes (LNs) compared with tumor-free satellite LNs and autologous PBMCs. These tumor-infiltrating $CD4^+CD25^{high}$ T cells express the Foxp3 transcription factor, display a phenotype of activated memory T cells, and inhibit in vitro the proliferation and cytokine production of autologous infiltrating $CD4^+CD25^-$ as well as $CD8^+$ T cells in a cell-contact-dependent manner. Thus, our data provide the first evidence that $CD4^+CD25^{high}$ T cells infiltrating human metastatic melanoma LNs behave as previously described $CD4^+CD25^{high}$ T_{reg}. In addition, we found that distinct subsets of T_{reg} may also operate locally.

Materials and Methods

Patients and samples

Twelve patients undergoing curative resections for LN metastatic melanoma before any immunotherapy or chemotherapy were included in this study after informed consent following human ethics committee procedures (Comité Consultatif pour la Protection des Personnes dans les Recherches Biomédicales; Hôpital Saint-Louis, Paris, France). Cancer stage was gathered according to the American Joint Committee on Cancer (41) (see Table I). Peripheral blood and LNs were collected at the time of surgery. Diagnosis of metastatic LNs was obtained by histological examination and immunochemical staining with PS100 and HMB45 Abs. LNs with large tumor invasion as well as LNs with no or minimal tumor invasion (corresponding to micrometastasis or only sinusal invasion by tumor cells) could be obtained. Blood samples from healthy donors were obtained from the Etablissement Français du Sang (Hôpital Saint-Louis). LN cell suspensions, obtained after sterile mechanical dissociation, were filtered, washed, and cryopreserved for further analysis.

Abs and flow cytometric analysis

The following Abs were used: PE anti-CD25 (M-A251), allophycocyanin anti-CD28 (CD28.2), allophycocyanin anti-CD69 (L78), allophycocyanin anti-CTLA-4(BN13), allophycocyanin anti-CD45RO (UCHL1), biotinylated anti-cutaneous lymphocyte-associated Ag (HECA-452), biotinylated anti-CD45RA (HI100), allophycocyanin anti-HLA-DR (Tü36), and PerCP streptavidin (all from BD Pharmingen, San Diego, CA). We also used: FITC anti-CD4 (clone S3.5; Caltag Laboratories, Burlingame, CA) and allophycocyanin anti-CD27 Abs (O323; eBioscience, San Diego, CA). Anti-CCR7 and anti-CXCR5 were kindly provided by Dr. M. Lipp (Max-Delbrück-Centrum, Berlin, Germany). For CTLA-4 intracellular staining, the Cytofix/Cytoperm kit was used according to the manufacturer recommendations (BD Pharmingen). Lymphocytes were gated according to their forward and size scatter characteristics, and four-color FACSCalibur analysis was performed using the CellQuest software (BD Biosciences, San Jose, CA).

Purification of $CD4^+CD25^{high}$, $CD4^+CD25^-$, and $CD8^+$ tumor-infiltrating lymphocytes

LN cell suspensions were coated with CD4 and CD8 magnetic microbeads (Miltenyi Biotec, Auburn, CA), together with FITC anti-CD4 and allophycocyanin anti-CD8 Abs (BD Pharmingen), according to the manufacturer recommendations, and isolated using LS separation columns on VarioMACS (Miltenyi Biotec). The positive fraction was then labeled with PE anti-CD25 Ab and the T cell populations were purified using a MoFlo Cell Sorter (DakoCytomation, Fort Collins, CO). Purities of $CD4^+CD25^{high}$, $CD4^+CD25^-$, and $CD8^+$ T cell populations were 92.4 ± 8.4%, 99 ± 1.3%, and 92.6 ± 6.1% (mean ± SD), respectively.

Proliferation assays

A total of 2.5×10^4 purified $CD4^+CD25^{high}$, $CD4^+CD25^-$, or $CD8^+$ T cells were cultured in triplicate with 5×10^4 allogeneic irradiated PBMCs in 96-well round-bottom plates in RPMI 1640 medium supplemented with 1 mM L-glutamine, 1% penicillin/streptomycin, nonessentials amino acid, sodium pyruvate (all from Invitrogen Life Technologies, Carlsbad, CA), and 10% FCS (Dominique Dutscher, Issy-les-Moulineaux, France). In inhibition assays, infiltrating $CD4^+CD25^-$ or $CD8^+$ T cells were cocultured with different numbers of infiltrating autologous $CD4^+CD25^{high}$ T cells. [^{3}H]Thymidine (ICN Biomedicals, Irvine, CA) was added at day 5 (1 μCi/well) for 16 additional hours before cell harvesting and counting in a Betaplate scintillation counter (LKB Pharmacia, Uppsala, Sweden). For blocking experiments, $CD4^+CD25^{high}$ were preincubated 20 min at 4°C with Abs before being cocultured in a 1 μg/ml Ab final concentration. The following blocking Abs were used: anti-CTLA-4 (BNI3; BD Pharmingen), anti-IL-10 (JES3-19F1; BD Pharmingen), anti-TGF-β_{1-3} (1D11; R&D Systems, Minneapolis, MN), and anti-glucocorticoid-induced TNFR (GITR; clone 110416; R&D Systems), and rat IgG2a,κ, mouse IgG2a,κ, and IgG1,κ immunoglobulins isotype controls (BD Pharmingen). For TCR stimulation, T cells were incubated with soluble anti-CD28 (CD28.2, 1 μg/ml; BD Pharmingen) in 96-well round-bottom plates previously coated overnight at 4°C with anti-CD3 (UCHT1, 0.5–1 μg/ml; Cymbus Biotechnology, Hants, U.K.). For transwell experiments, $CD4^+CD25^-$ were placed at the bottom of a 96-well round-bottom plate together with irradiated allogeneic PBMCs, and were separated by a 0.2-μm size anopore membrane (Nunc, Roskilde, Denmark) from $CD4^+CD25^{high}$ T cells placed together with irradiated allogeneic PBMCs in the top chamber of the transwell. At day 5, the transwell was removed and [^{3}H]thymidine incorporation was measured as described above.

Cytokines production

ELISA was used according to manufacturer recommendations (OptEIA; BD Pharmingen) to detect IL-2, IL-4, IL-10, TGF-β_1, and IFN-γ in supernatants of T cell cultures after 5 days of allogeneic stimulation or 3 days of anti-CD3/anti-CD28 stimulation. The minimal levels of detection were 3.9 pg/ml for IL-2, IL-4, and IL-10, 2.3 pg/ml for IFN-γ, and 31.2 pg/ml for TGF-β in four-time diluted supernatants.

Quantitative real-time PCR

RNA were first purified using the RNeasy kit (Qiagen, Carlsbad, CA), and oligo(dT) or random hexanucleotide-primed first strand cDNAs were synthesized using SuperScript II reverse transcriptase (Invitrogen Life Technologies). They were then used as a template for real-time PCR, with the TaqMan Universal Master Mix (Applied Biosystems, Foster City, CA). Specific primers for Foxp3 and human hypoxanthine ribosyltransferase 1 (HPRT) were used with the following TaqMan MGB probe (Assays-on-demand gene expression assays; Applied Biosystems): 5′-6FAM-ATCCGCT GGGCCATCCTGGAGGCTC-3′ (FoxP3), and 5′-6FAM-GGTCAAGGT CGCAAGCTTGCTGGTG (HPRT). Because sequences for the specific unlabelled PCR primers were not provided by the manufacturer, the real-time PCR products for Foxp3 were controlled by sequencing analysis, using an ABI Prism 3700 detection system (Applied Biosystems). Real-time PCR was conducted in a GeneAmp 5700 detection system (Applied Biosystems), with the following cycling conditions: 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, and 1 min at 60°C. The mean values from duplicates were used for calculations. Data are expressed as normalized expression obtained by dividing the relative cDNA level for each sample by the relative cDNA level of HPRT for the same sample, where HPRT = 1.

T cell repertoire diversity

TCR Vβ chain repertoire of sorted infiltrating $CD4^+CD25^{high}$ and $CD4^+CD25^-$ T cells was analyzed by quantitative Immunoscope (Applied Biosystems), as previously described (42, 43). cDNAs obtained after reverse transcription of total RNA with oligo$(dT)_{17}$ and SuperScript II reverse transcriptase (Invitrogen Life Technologies) were amplified with each of 24 TCR Vβ subfamily-specific primers and a nested fluorochrome-labeled TaqMan probe for TCR Cβ. Real-time PCR was conducted in a GeneAmp 5700 detection system (Applied Biosystems). PCR products were used as templates in run-off reactions using a nested TCR Cβ fluorescent primer producing labeled ssDNA fragments that were separated on a denaturing 6% acrylamide gel on a 373 DNA sequencer (Applied Biosystems) and analyzed with Immunoscope software (42).

Statistical analysis

Statistical analysis was performed using paired or unpaired Student's *t* tests with the Kaleidagraph software (Synergy Software, Reading, PA).

Results

Overrepresentation of $CD4^+CD25^{high}$ in metastatic melanoma LNs

Because regulatory $CD4^+$ T cell activity has been identified in the $CD4^+CD25^{high}$ T cell subpopulation in human PBMCs and thymocytes (24, 25, 27, 28, 30–33), we first estimated the proportion of such $CD4^+CD25^{high}$ T cells among lymphocytes infiltrating metastatic LNs in melanoma patients. Our study included 12 patients suffering from stage III melanoma before any treatment other than curative surgical resection (Table I). Isolated lymphocytes were gated to estimate the proportion of $CD4^+$ T cells that expressed high level of CD25 (IL-2R α-chain) and lower level of CD4 as previously described for $CD4^+CD25^+$ T_{reg} (Fig. 1*A*).

The frequency of $CD4^+CD25^{high}$ T cells among $CD4^+$ T cells was 11.06 ± 1.7% (mean ± SD) in metastatic LNs (range 8.3–14.05%; $n = 13$). We also studied tumor-free LNs and found that the frequency of $CD4^+CD25^{high}$ T cells was 6.2 ± 4.2% ($n = 16$). A global comparison between metastatic LNs and tumor-free LNs revealed almost a 2-fold increased frequency in metastatic LNs ($p = 0.0006$). The frequency of $CD4^+CD25^{high}$ T cells in metastatic LNs was also significantly increased when compared with the frequency observed in autologous PBMCs (4.9 ± 1.9%, range 2.6–8.7%, $n = 8$, $p < 0.0001$) or in healthy donor PBMCs (5.8 ± 1.7%, range 3–8.2%, $n = 10$, $p < 0.0001$). No statistical difference was found between frequencies of $CD4^+CD25^{high}$ T cells in PBMCs from metastatic melanoma patients and healthy donors ($p = 0.37$). When performing matched sample analysis (i.e., comparing metastatic LNs with either PBMCs or tumor-free LNs from the same patient), the difference remained highly significant ($p = 0.014$ and $p = 0.016$, respectively).

Altogether, these results revealed that the proportion of $CD4^+CD25^{high}$ T cells is clearly increased in metastatic LNs compared with tumor-free LNs and autologous PBMCs.

Infiltrating $CD4^+CD25^{high}$ T cells display a memory and activated phenotype

The majority of $CD4^+CD25^{high}$ T cells in 11 metastatic LNs from 10 patients expressed CD45RO (73.2 ± 11.8%) and few were CD45RA positive (18.9 ± 8.5%). They all expressed CD27 and CD28 molecules (Fig. 1*B*) and were mostly CCR7 negative (87 ± 6.3%). Thus, most of these $CD4^+CD25^{high}$ T cells belong to the $CD45RA^-/CD27^+$ conventional memory $CD4^+$ T cell population as defined by Campbell and colleagues (44). Few expressed HLA-DR (6.6 ± 2.5%) or the cutaneous homing molecule cutaneous lymphocyte-associated Ag (13.1 ± 11.2%), and the great majority were CXCR5 negative (95 ± 4.7%) (Fig. 1*B*, and data not shown). As reported for human circulating T_{reg}, the majority of infiltrating $CD4^+CD25^{high}$ T cells intracellularly expressed the CTLA-4 molecule. However, more $CD4^+CD25^{high}$ T cells expressed CTLA-4 in metastasis (75.5 ± 15.8%) compared with autologous PBMCs (48.9 ± 15%) and their levels of expression were significantly higher (mean fluorescence intensity (MFI) = 44 ± 26 vs 15 ± 8, respectively, $p = 0.01$) (Fig. 1*C*). Furthermore, surface expression of CTLA-4 was found on LN-infiltrating $CD4^+CD25^{high}$ T cells (4.5 ± 2.4%), but not on their circulating counterparts (0.7 ± 0.35%). Such an intracellular and surface up-regulation of CTLA-4 has been reported for human T_{reg} after activation in vitro (28), supporting the idea that infiltrating $CD4^+CD25^{high}$ T cells were activated at the tumor site.

Indeed, infiltrating $CD4^+CD25^{high}$ T cells expressed high levels of the activation molecule CD69 (43.8 ± 16%, range 19–71%) compared with only 1.2 ± 1.2% and 0.5 ± 0.4% in 8 autologous or 10 healthy donor PBMCs, respectively. Along the same lines, CD27, which is known to be up-regulated after TCR- or CD3-cross-linking (45), was expressed at a higher level on infiltrating $CD4^+CD25^{high}$ T cells compared with circulating $CD4^+CD25^{high}$ T cells (MFI = 754 ± 149 vs 489 ± 159, $p = 0.002$), also arguing for the activation of infiltrating $CD4^+CD25^{high}$ T cells. Moreover and as recently described for murine LNs (46), we also found activated $CD4^+CD25^{high}$ T cells expressing high levels of CTLA-4 (61.2 ± 18.9%, MFI = 32.7 ± 19) and CD69 (48.6 ± 16.9%) in 16 tumor-free LNs from seven different patients, as well as in two cutaneous draining LNs from healthy donors (data not shown).

Thus, $CD4^+CD25^{high}$ T cells present in metastatic as well as in tumor-free LNs display an activated phenotype.

Infiltrating $CD4^+CD25^{high}$ T cells express the Foxp3 transcription factor

Foxp3 was recently described as an important transcription factor involved in the development and function of murine $CD4^+CD25^+$ T_{reg} and appears to be the most specific molecular marker available to date (21). We first tested PBMCs from three healthy donors using real-time quantitative PCR. As expected, $CD4^+CD25^{high}$ T cells expressed high levels of Foxp3 while $CD4^+CD25^-$, $CD4^+CD25^{int}$ and $CD8^+$ T cells expressed only very low levels (Fig. 2, and data not shown). Similarly, when analyzing Foxp3 expression in nine melanoma metastatic LNs from seven different

Table I. *Characteristics of the melanoma patients studied*[a]

Patients	Sex	Age (year)	Site of LNs	LNs Dissection	Tumor Stage	Disease Interval
SLM2	M	56	Cervical	1 N^+/4 N	IIIb/IIIc	2 year
SLM6	F	55	Axillary	2 N^+/3 N	IIIb/IIIc	Unknown
SLM8	M	35	Inguino-iliac	1 N^+/15 N	IIIb/IIIc	4 year
SLM11	F	63	Inguino-iliac	4 N^+/12 N	IIIc	5 year
SLM12	F	59	Inguino-iliac	1 N^+/8 N	IIIb/IIIc	4 year
SLM14	M	81	Cervical	1 N^+/13 N	IIIc	6 mo
SLM15	F	64	Inguino-iliac	5 N^+/17 N	IIIc	15 mo
SLM16	M	73	Axillary	2 N^+/21 N	IIIb/IIIc	21 mo
SLM17	M	64	Inguino-iliac	12 N^+/24 N	IIIc	6 mo
SLM18	M	53	Inguino-iliac	3 N^+/12 N	IIIc	0
SLM19	M	23	Axillary	1 N^+/11 N	IIIb/IIIc	Unknown
SLM20	F	55	Inguino-iliac	7 N^+/17 N	IIIc	13 year

[a] Twelve stage III melanoma patients with metastatic LNs were included in this study. The anatomical site of surgical LN resection and the results of histopathological examinations are indicated (N, total LNs resected; N^+, (metastatic LNs resected). Tumor stages were determined according to the American Joint Commitee for Cancer (41). Disease intervals between the removals of the primary cutaneous melanoma and of the metastatic LNs are given. For two patients (SLM6 and SLM19), the primary cutaneous lesion was not evidenced. For another one (SLM18) the primary melanoma and the metastatic LNs were discovered concomitantly.

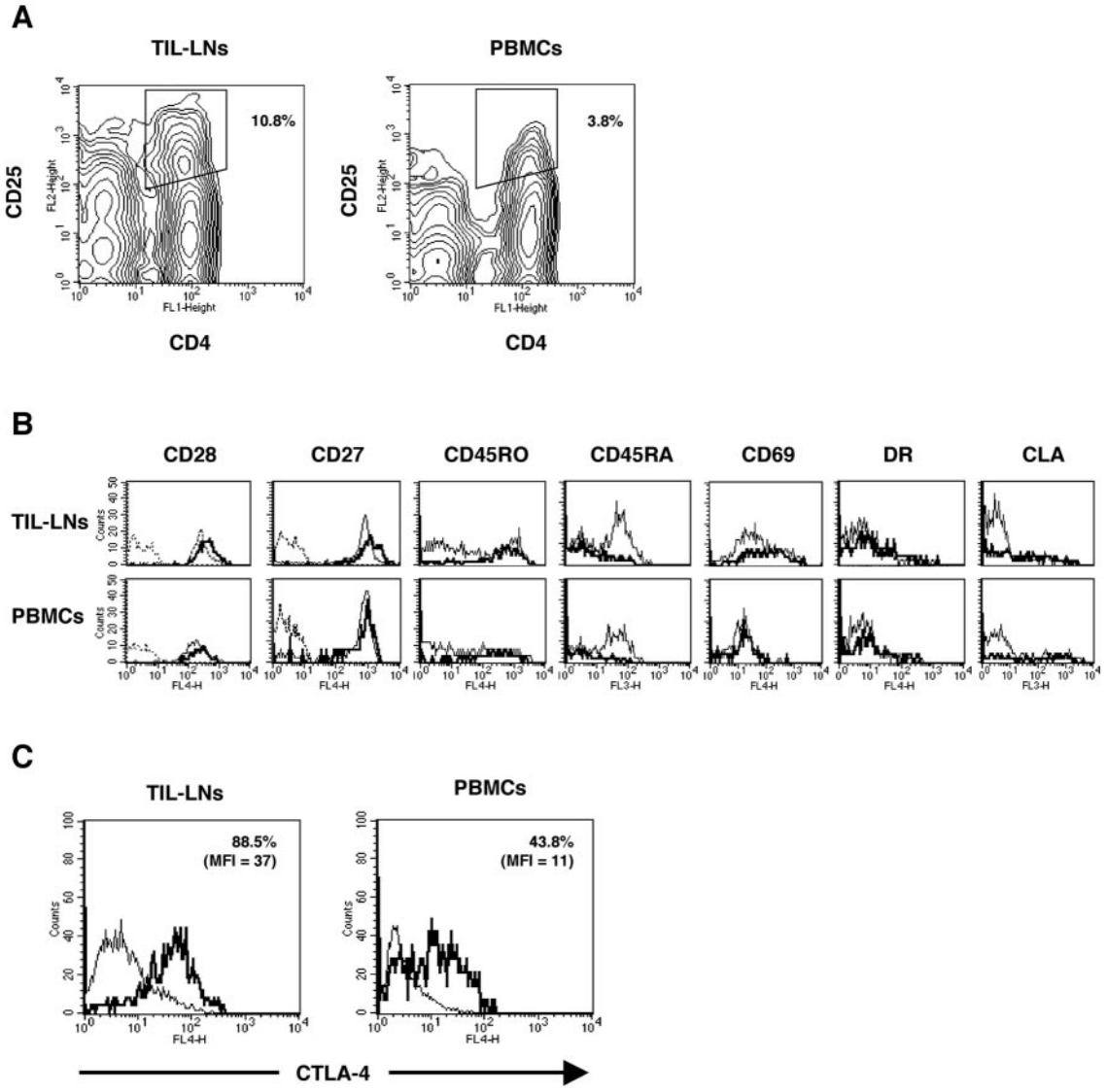

FIGURE 1. $CD4^+CD25^{high}$ T cells are overrepresented in melanoma metastatic LNs and display a memory and activated phenotype. *A*, Infiltrating LN lymphocytes (TIL-LNs) and autologous PBMCs were stained with FITC anti-CD4 and PE anti-CD25 Abs, and $CD4^+CD25^{high}$ T cells were gated as indicated. A representative case is shown with increased percentage of $CD4^+CD25^{high}$ T cells among infiltrating $CD4^+$ cells in LNs compared with PBMCs. *B*, FACS analysis of $CD4^+CD25^{high}$ (thick lines) and $CD4^+CD25^-$ T cells (thin lines) among TIL-LNs and autologous PBMCs showing that infiltrating $CD4^+CD25^{high}$ T cells display a memory and activated phenotype. *C*, Intracellular expression of CTLA-4 in $CD4^+CD25^{high}$ T cells (thick lines) and $CD4^+CD25^-$ T cells (thin lines) among TIL-LNs and autologous PBMCs. A representative case is shown revealing that infiltrating $CD4^+CD25^{high}$ T cells express higher levels of intracellular CTLA-4 compared with $CD4^+CD25^{high}$ T cells from PBMCs. Percentages of CTLA-4-positive T cells among $CD4^+CD25^{high}$ T cells and MFI are indicated.

patients, we found that infiltrating $CD4^+CD25^{high}$ T cells highly expressed Foxp3 while $CD4^+CD25^-$ T cells expressed very low levels (Fig. 2). The levels of Foxp3 expression in $CD4^+CD25^{high}$ T cells from metastatic LNs compared with autologous or healthy donor PBMCs were almost similar (Fig. 2). In three tumor-free LNs from different patients, $CD4^+CD25^{high}$ T cells also expressed Foxp3 (data not shown). Altogether, these data revealed that $CD4^+CD25^{high}$ T cells infiltrating metastatic melanoma as well as tumor-free LNs express the Foxp3 transcription factor, strongly suggesting that this population includes T_{reg}.

Infiltrating $CD4^+CD25^{high}$ T cells suppress in vitro the proliferation and cytokine production of infiltrating $CD4^+CD25^-$ as well as $CD8^+$ T cells

We next evaluated the proliferative capacities and the regulatory function of $CD4^+CD25^{high}$ T cells from metastatic melanoma LNs. For this purpose, we sorted $CD4^+CD25^{high}$ as well as $CD4^+CD25^-$ and $CD8^+$ T cells, and tested them in in vitro proliferation assays. In all the nine metastatic LNs tested from six different patients, the sorted $CD4^+CD25^{high}$ T cells proliferated poorly and did not produce IL-2 or IFN-γ in response to allogeneic stimulation (Figs. 3, *A*, *D*, and *E*), thus exhibiting an anergic profile similar to what has been described for human T_{reg} (24, 25, 27, 28, 30–33). In contrast, $CD4^+CD25^-$ T cells proliferated vigorously and produced both IL-2 and IFN-γ in response to allogeneic stimulation (Fig. 3, *A* and *D*, and data not shown).

Importantly, the proliferation of $CD4^+CD25^-$ T cells was strongly inhibited in a dose-dependent manner when cocultured with $CD4^+CD25^{high}$ T cells (Fig. 3, *A* and *B*). The median percentage of inhibition at a 1:1 $CD4^+CD25^{high}$:$CD4^+CD25^-$ T cell ratio obtained in nine metastatic LNs was 77.5% (range 51–98%) and remained detectable even at a 1:8 or 1:10 ratio in most of the

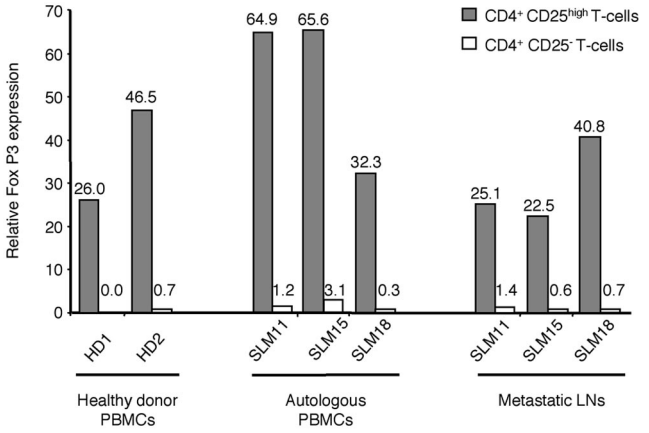

FIGURE 2. Infiltrating $CD4^+CD25^{high}$ T cells constitutively express Foxp3. cDNAs obtained from sorted $CD4^+CD25^{high}$ and $CD4^+CD25^-$ T cells were submitted to quantitative real-time PCR with specific primers and probes for Foxp3 and HPRT. Relative Foxp3 expression for each sample is shown after normalization to HPRT expression, where HPRT = 1. Results obtained from representative healthy donor PBMCs, autologous PBMCs, and metastatic LNs are shown, enlightening that in all compartments $CD4^+CD25^{high}$ T cells, but not $CD4^+CD25^-$ T cells, express substantial level of Foxp3. The healthy donors and melanoma patients are identified by the abbreviation HD or SLM, respectively.

five cases where all ratios were tested (Fig. 3, *A* and *B*). In addition, $CD4^+CD25^{high}$ T cells strongly inhibited IL-2 and IFN-γ production of $CD4^+CD25^-$ T cells (Fig. 3*D*, and data not shown) corresponding to a 95.5 $\pm$ 6.6% of inhibition for IL-2 and 98.6 $\pm$ 3.8% for IFN-γ at a 1:1 ratio. It is noteworthy that at 1:8 or 1:10 ratios, which almost correspond to the ratios observed in metastatic LNs, the inhibition of cytokine production was still major (~90% inhibition; Fig. 3*C*, and data not shown), while the inhibition of proliferation was weaker (~30% inhibition; Fig. 3*A*). This suggests that infiltrating $CD4^+CD25^{high}$ T cells may be more suppressive on T cell functions than on T cell proliferation. In four metastatic LNs, the suppressive activity of $CD4^+CD25^{high}$ T cells was also tested at a 1:1 ratio against sorted infiltrating $CD8^+$ T cells. Although these cells proliferated to a lower extent than $CD4^+CD25^-$ T cells in response to allogeneic stimulation, their proliferation was also strongly inhibited by $CD4^+CD25^{high}$ T cells in a dose-dependent manner with a median percentage of inhibition of 62% at a 1:1 ratio (Fig. 3*B*, and data not shown). Similarly, the IFN-γ production of $CD8^+$ T cells was also strongly inhibited by $CD4^+CD25^{high}$ T cells (Fig. *3E*), with a median percentage of inhibition of 85% at a 1:1 ratio. In two LNs with minimal tumor invasion, the inhibition of proliferation of both $CD4^+CD25^-$ and $CD8^+$ T cells was weak, around 50% at 1:1 ratio (data not shown). In addition, as expected from recent studies on murine LNs (46), $CD4^+CD25^{high}$ T cells isolated from four tumor-free LNs from three different patients also displayed some suppressive activity, albeit not as potent as in metastatic LNs, because suppression was not detected at a lower ratio than 1:2 or 1:4 (Fig. *3C*).

Taken together, $CD4^+CD25^{high}$ T cells infiltrating melanoma metastatic LNs, and to a lesser extent tumor-free LNs, suppressed in vitro the proliferation and cytokine production of both infiltrating $CD4^+CD25^-$ as well as $CD8^+$ T cells.

Suppression by tumor-infiltrating $CD4^+CD25^{high}$ T cells is mediated through a cell contact mechanism

Although controversial, the mechanism of action of human $CD4^+CD25^{high}$ T_{reg} appears to be largely cell contact dependent (24, 25, 27, 28, 30–33). The role of cytokines, like IL-10 or TGF-β, as well as cell surface molecules, like CTLA-4 or GITR, is still a matter of debate (14). Therefore, we investigated whether infiltrating $CD4^+CD25^{high}$ T cells inhibited cell proliferation in vitro through a cell-contact- and/or cytokine-dependent mechanism. Transwell experiments performed in four metastatic LNs revealed that $CD4^+CD25^{high}$ T cells required cell contact to suppress proliferation of $CD4^+CD25^-$ T cells, because in all cases the inhibition of proliferation was either completely or partially abolished by the transwell (Fig. 4*A*, and data not shown). Preincubation of $CD4^+CD25^{high}$ T cells with blocking anti-IL-10 or anti-TGF-β_{1-3} Abs significantly reverted the suppression in experiments performed in three cases, while isotype control blocking mAbs did not (Fig. 4*A*, and data not shown). As control, the preincubation of $CD4^+CD25^-$ T cells alone with these blocking Abs did not affect their proliferation (data not shown). Thus, the suppressive activity of LN-infiltrating $CD4^+CD25^{high}$ T cells was found to be both contact and cytokine dependent. Interestingly, we found that preincubation of infiltrating $CD4^+CD25^{high}$ T cells with blocking anti-CTLA-4 Ab also significantly reverted the suppression (Fig. 4*A*). In all the cases tested, the anti-GITR Ab did not significantly alter the suppressive activity of the $CD4^+CD25^{high}$ T cells (Fig. 4*A*). Finally and as previously described (27, 30–32), addition of IL-2 (50 or 100 IU/ml) allowed $CD4^+CD25^{high}$ T cells to proliferate and abolished their suppressive activity (data not shown).

Therefore, $CD4^+CD25^{high}$ T cells infiltrating melanoma metastatic LN suppress $CD4^+CD25^-$ T cells in a cell-contact- and CTLA-4-dependent mechanism. In addition, a cytokine-dependent mechanism could also be involved.

Tumor-infiltrating $CD4^+CD25^{high}$ but also $CD4^+CD25^-$ T cells secrete IL-10 and/or TGF-β

Because the suppressive activity of $CD4^+CD25^{high}$ T cells was abolished by anti-IL-10 or anti-TGF-β_{1-3} blocking Abs, we investigated whether infiltrating $CD4^+CD25^{high}$ and $CD4^+CD25^-$ T cells indeed produced these cytokines upon in vitro stimulation. We found that $CD4^+CD25^{high}$ T cells from metastatic LNs with large tumor invasion produced IL-10 upon allogeneic stimulation (36.6 up to 279.2 pg/ml; mean, 110.8 pg/ml), while in cases of minimal tumor invasion such a production was not detected (Fig. 4*B*). Because in these latter cases a lower level of inhibition of $CD4^+CD25^-$ T cell proliferation was also observed compared with more advanced disease, this suggests that IL-10 production may be correlated to the strength of suppression detected in vitro (Figs. 3*B* and 4*B*). However, stronger TCR-mediated stimulation using coated anti-CD3 and soluble anti-CD28 Abs revealed that these cells also displayed the capacity to secrete IL-10 (data not shown). In contrast, infiltrating $CD4^+CD25^-$ T cells also secrete IL-10 (27.9 up to 265.9 pg/ml; mean, 130.9 pg/ml) upon allogeneic stimulation, mostly in case of large tumor invasion (Fig. 4*B*). In tumor-free LNs, we similarly observed that both $CD4^+CD25^{high}$ and $CD4^+CD25^-$ T cells could produce IL-10, albeit at slightly lower levels (mean, 84 and 69 pg/ml, respectively).

In few cases, infiltrating $CD4^+CD25^{high}$ T cells and $CD4^+CD25^-$ T cells were found to produce TGF-β_1 at a substantial level (>2000 and up to 8500 pg/ml) (Fig. 4*C*). In the case of minimal tumor invasion, such a TGF-β_1 secretion was not observed and could not be induced by stimulation with anti-CD3 and anti-CD28 Abs (data not shown). Finally, TGF-β_1 production was not detected when analyzing T cells from tumor-free LNs (data not shown).

The fact that infiltrating $CD4^+CD25^-$ T cells secreted IL-10 and/or TGF-β_1 suggested that they may also display some suppressive activity. Indeed, when tested functionally in coculture experiments with infiltrating $CD8^+$ T cells, irradiated $CD4^+CD25^-$

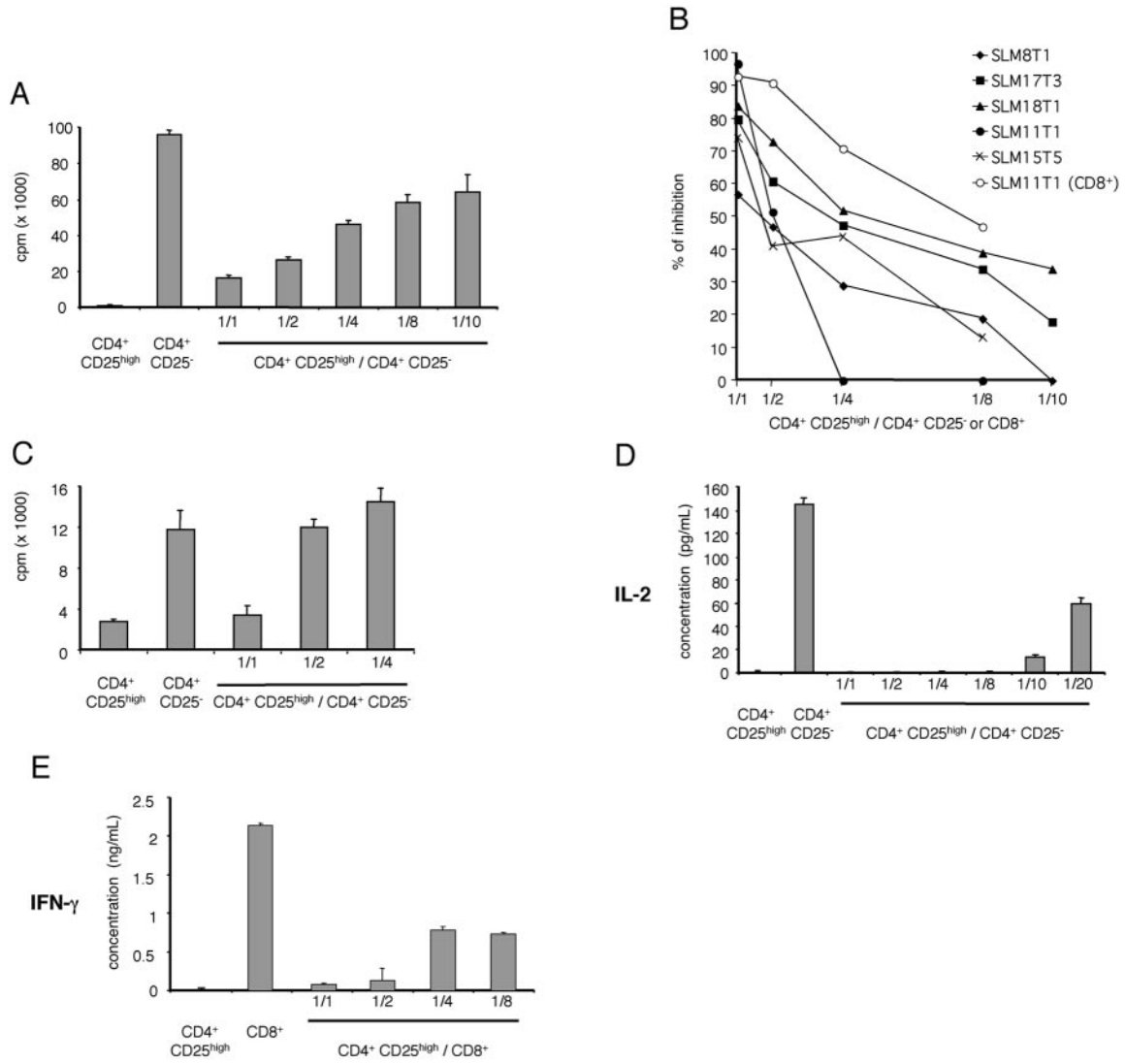

FIGURE 3. Infiltrating $CD4^+CD25^{high}$ T cells inhibit the proliferation and cytokine production of corresponding $CD4^+CD25^-$ and $CD8^+$ T cells. *A*, Sorted infiltrating $CD4^+CD25^{high}$ and $CD4^+CD25^-$ T cells were cultured either alone or at different $CD4^+CD25^{high}$:$CD4^+CD25^-$ ratios under allogeneic stimulation. [^{3}H]Thymidine incorporations at day 5 are shown for one representative patient. *B*, Infiltrating $CD4^+CD25^{high}$ T cells inhibit the proliferation of autologous infiltrating $CD4^+CD25^-$ (filled symbols) or $CD8^+$ T cells (open symbols) in a dose-dependent manner. Percentages of inhibition of the proliferation at different ratios are shown for five distinct metastatic LNs from different patients. *C*, Sorted $CD4^+CD25^{high}$ T cells infiltrating tumor-free LN also display some suppressive activity but at a lesser extent than in metastatic LN from the same patient (shown in *A*). IL-2 production of infiltrating $CD4^+CD25^-$ T cells (*D*) and IFN-γ production of infiltrating $CD8^+$ T cells (*E*) are suppressed when cocultured with $CD4^+CD25^{high}$ T cells at different ratios. Data obtained by ELISA on culture supernatants for a representative metastatic LN are shown.

T cells suppressed the proliferation and IFN-γ production of $CD8^+$ T cells (data not shown).

Altogether, these data indicate that both infiltrating $CD4^+CD25^{high}$ and $CD4^+CD25^-$ T cells can produce the immunosuppressive cytokines IL-10 and/or TGF-β_1, and that IL-10 may play a role in the suppressive activity of $CD4^+CD25^{high}$ T cells. They also provide evidence that cells with a Tr1/Th3-like phenotype (22, 23) are also present in melanoma metastatic LNs, mostly in cases of large tumor invasion. Finally, because both infiltrating $CD4^+CD25^{high}$ and $CD4^+CD25^-$ T cells did not produce IL-4 upon stimulation (data not shown), the presence of Th2 cells is unlikely.

Tumor-infiltrating $CD4^+CD25^{high}$ T cells display a largely polyclonal TCR Vβ (TCRBV) repertoire

It has been largely described in mice and humans that oligoclonality of TCR usage and characteristic features of complementarity-determining regions 3 (CDR3) regions may reveal Ag-expanded T cells in a given T lymphocyte population even if the Ag is not known (47). Thus, to obtain insight into the potential Ag-driven expansion of $CD4^+CD25^{high}$ T cells infiltrating melanoma metastatic LNs, we performed a detailed Immunoscope analysis of their TCRBV repertoire by, first, quantifying the BV family usage through real-time PCR, and, second, by determining their CDR3β chain length distributions through Immunoscope analysis (42, 43). We found that both $CD4^+CD25^{high}$ and $CD4^+CD25^-$ T cell populations from three metastatic LNs from different patients and from one tumor-free LN expressed an highly diverse set of BV families and that these populations differed quantitatively only on a limited number of BV families for a given patient. Similar results were obtained for the four LNs studied and a representative case is shown in Fig. 5*A*. In each case, including the tumor-free LN, similar BV families (as BV15, 20, or 23, for example) were poorly or not detected in both $CD4^+CD25^{high}$ and $CD4^+CD25^-$ T cell populations. They correspond to infrequently used BV families in the

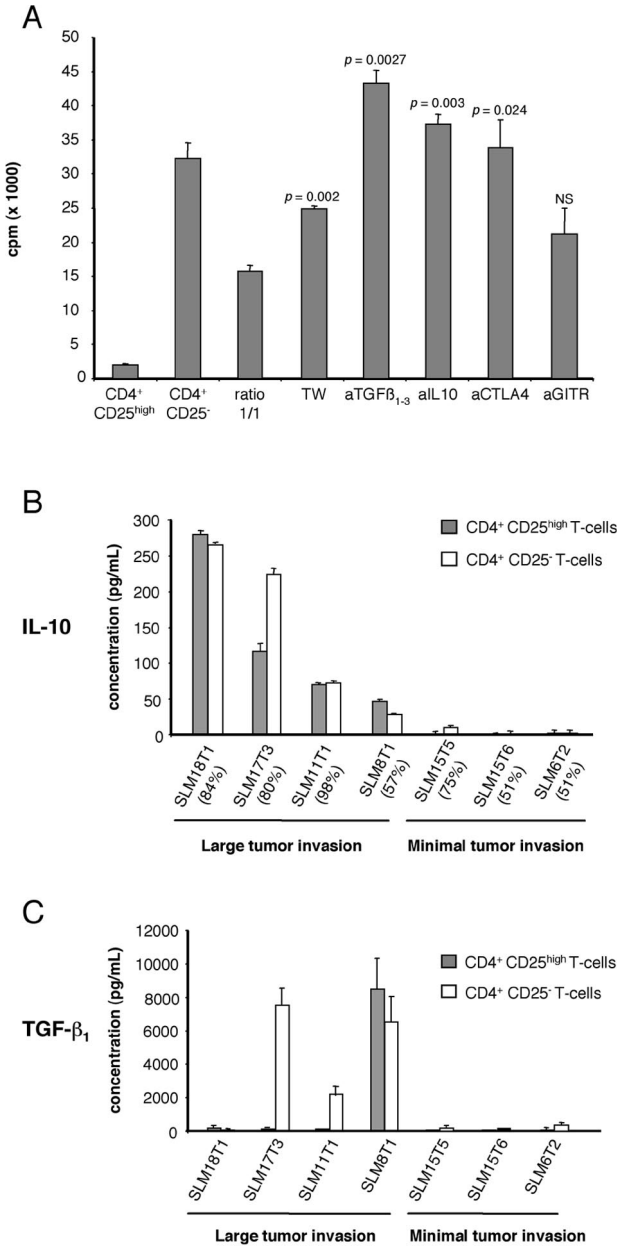

FIGURE 4. Cell-contact- and cytokine-dependent suppression activity of infiltrating $CD4^+CD25^{high}$ T cells could be identified. *A*, [^{3}H]Thymidine incorporation of infiltrating $CD4^+CD25^{high}$ and $CD4^+CD25^-$ T cells is shown for a representative metastatic LN under allogeneic stimulation at a 1:1 ratio separated or not by a transwell (TW) or in the presence of blocking mAbs: anti-TGF-β_{1-3} (aTGFβ_{1-3}), anti-IL-10 (aIL-10), anti-CTLA-4 (aCTLA-4), or anti-GITR (aGITR). Suppressive activity of infiltrating $CD4^+CD25^{high}$ T cells was found to be inhibited by both TW and blocking Abs. Statistical significative *p* values when compared with the 1:1 ratio are indicated. IL-10 (*B*) and TGF-β_1 (*C*) production of infiltrating $CD4^+CD25^{high}$ and $CD4^+CD25^-$ T cells were assessed by ELISA on supernatants after allogeneic stimulation. Infiltrating $CD4^+CD25^{high}$ but also $CD4^+CD25^-$ T cells from LNs with advanced tumor involvement produced IL-10 and occasionally TGF-β_1, while cells from less-invaded LNs did not. The percentage of inhibition of the proliferation of $CD4^+CD25^-$ T cells by infiltrating $CD4^+CD25^{high}$ T cells obtained at a 1:1 ratio as shown in Fig. 3*B* is indicated above each tumor name in *B*.

normal human TCR repertoire (42). When looking at the CDR3β chain length profiles (Fig. 5*B*), both populations showed an almost Gaussian-like curve distribution for each BV family studied with no evidence for oligoclonal expansion even in case of differential usage of a given BV (see for example the BV2 profile among the representative BV profiles shown in Fig. 5*B*).

Overall, these data do not argue for a restricted Ag-driven expansion of $CD4^+CD25^{high}$ T cells infiltrating melanoma metastatic LNs.

Discussion

$CD4^+CD25^{high}$ T_{reg} have been shown in murine models to play an important role in dominant peripheral tolerance by controlling potentially harmful autoreactive T cells. They have been also implicated recently in the down-regulation of antitumor responses in transplantable murine tumor models (15–17). Such a role could explain the poor clinical efficiency of immunotherapeutic protocols in human tumors. Recently, it was shown that $CD4^+CD25^{high}$ T_{reg} specifically express a transcription factor, Foxp3, which plays a major role in their development and function (18–21, 26, 29). Foxp3 is currently considered to be the most accurate marker to identify T_{reg} (21). Indeed, in human PBMCs, we found that Foxp3 is expressed almost exclusively by the $CD4^+CD25^{high}$ T cell population that is known to contain T_{reg} (27, 28, 30, 31). In the present study of melanoma metastatic LNs, we identified substantial Foxp3 expression among infiltrating $CD4^+CD25^{high}$ T lymphocytes in all the cases studied. Furthermore, such infiltrating $CD4^+CD25^{high}$ T cells display the phenotype of peripheral memory T cells ($CD45RO^+CD27^+$) (44) and constitutively express high levels of CD152 (CTLA-4). Functionally, $CD4^+CD25^{high}$ T cells do not proliferate in vitro and do not produce IL-2 or IFN-γ upon TCR-mediated activation. They clearly suppress both the proliferation and cytokine production of infiltrating $CD4^+CD25^-$ as well as $CD8^+$ T cells in a cell-contact-dependent manner. All these features described above have been recently ascribed to human peripheral T_{reg} (24, 25, 27, 28, 30–33). The fact that these cells widely inhibit both infiltrating $CD4^+CD25^-$ as well as $CD8^+$ T cells is in accordance with the observation that once activated, both mice and human T_{reg} suppress other T cells in an Ag-independent manner (13, 14). Thus, our data reveal that $CD4^+CD25^{high}$ T cells found in melanoma metastatic LNs behave as T_{reg}, suggesting that they may play an active role in down-regulating local antitumor responses. Such a role for suppressive T cells has been suggested long time ago in melanoma by Mukherji and colleagues (48, 49), who observed $CD4^+$ T cell clones with some suppressive activity from metastatic LNs. We have preliminary evidence that these infiltrating $CD4^+CD25^{high}$ T cells could inhibit in vitro the proliferation of a $CD8^+$ T cell clone recognizing the tumor Ag MelanA/Mart-1. However, as mentioned above, such an observation is not surprising due to the suppressive activity of T_{reg} independent of the specificity of the target effectors (13, 14).

Importantly, we found that such $CD4^+CD25^{high}$ T_{reg} are over-represented in metastatic LNs and express high levels of CTLA-4, CD69, and CD27, consistent with the phenotype of activated T_{reg}. This directly raises the question whether these cells are recruited and expanded at the tumor site from circulating T_{reg} or from resident LN T_{reg}. It was recently demonstrated by Fisson et al. (46) that in mice, a fraction of T_{reg} present in normal LNs and spleen is continuously activated and is dividing in the steady-state while another fraction remains quiescent. In addition, according to what was suspected (14), Salomon's group have nicely shown that activation of LN T_{reg} is driven by physiological sustained presentation of tissue self-Ags in draining LNs (46). In our case, we found the presence of activated T_{reg} in metastatic LNs, but also in tumor-free satellite LNs, as well as in two cutaneous draining LNs from healthy donors. In the cases of tumor-free and normal LNs, the

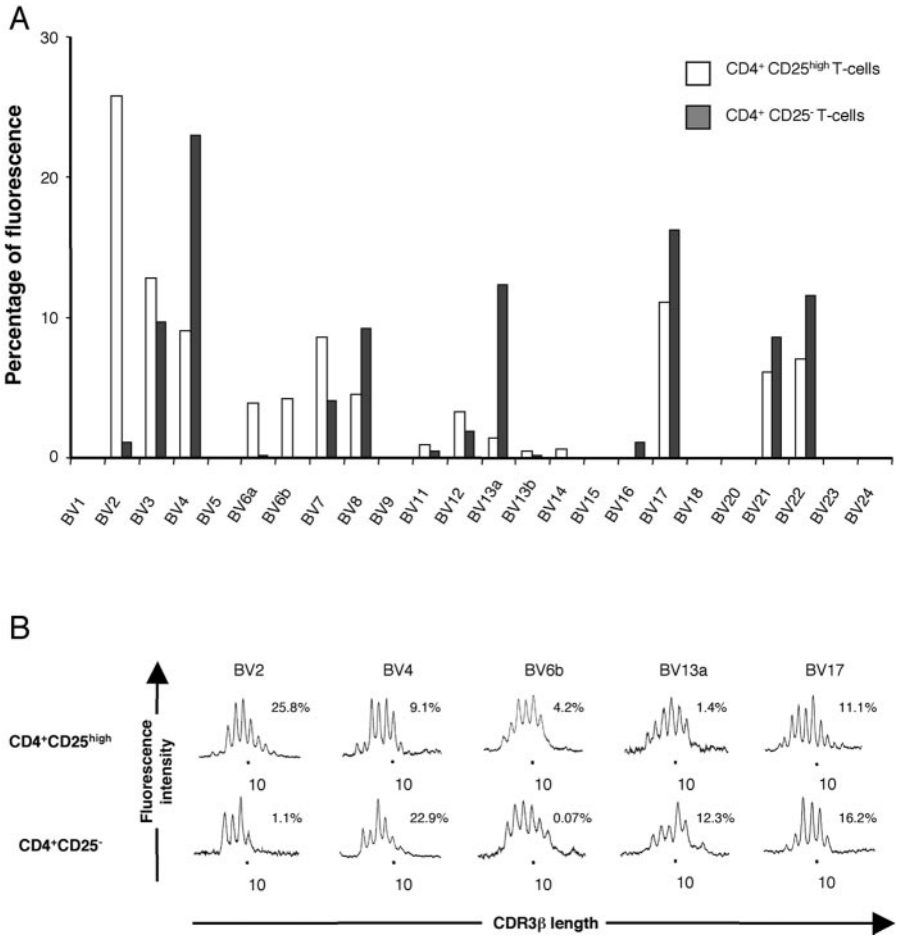

FIGURE 5. Infiltrating $CD4^+CD25^{high}$ T cells display a polyclonal TCRBV repertoire. *A*, Real-time PCR amplifications analyzing the BV usage of TCR β-chains expressed by sorted infiltrating $CD4^+CD25^{high}$ and $CD4^+CD25^-$ T cells. The percentages obtained for BV1 to BV24 subfamily usage show that both populations express a highly diverse set of BV subfamilies with few differences from each other. *B*, CDR3 length distribution of TCRBV chains expressed by infiltrating $CD4^+CD25^{high}$ (*upper line*) and $CD4^+CD25^-$ T cells (*lower line*) for selected representative BV subfamilies. For each Immunoscope profile, fluorescent intensity in arbitrary units, represented as a function of the CDR3 length measured in codons, reveals an almost Gaussian distribution with no evidence for oligoclonal expansion. The peak of the 10th codon is marked on the abscissa axis. Percentages indicate the usage of given BV, as derived from quantitative amplifications. Analysis of a representative metastatic LN is shown in *A* and *B*.

observed activated T_{reg} could well correspond to such T_{reg} activated at the steady-state because they express Foxp3 (data not shown). In the case of metastatic LNs, the observed expansion of the T_{reg} already present locally may be due to an increased Ag stimulation either by tumor-specific Ags or self-Ags expressed by tumor cells. In accordance with this hypothesis, the fact that $CD4^+CD25^{high}$ T cells in both metastatic and tumor-free LNs express a largely polyclonal repertoire does not argue for a stimulation by a limited number of Ags, but rather for a stimulation by a large panel of Ags. However, we cannot exclude that such an expansion is due to nonspecific stimulation of steady-state T_{reg} in the context of a local suppressive microenvironment generated by invading tumor cells via immunosuppressive cytokines (as TGF-β or IL-10). To solve these issues of the Ag specificities of the T_{reg} expanded at the tumor site, further studies analyzing isolated infiltrating T_{reg} clones are needed. In favor of an Ag-driven stimulation of infiltrating T_{reg} in melanoma, Wang's group (50) recently identified the tumor Ag LAGE-1 as the ligand recognized by some infiltrating regulatory T cell clones isolated not from metastatic LNs but from a melanoma tumor.

It is important to note that the existence of such a steady-state level of activation of LN T_{reg} may well control the occurrence of any efficient antitumor immune response during the course of tumor development, even prior to tumor cell invasion, when tumor Ags from the primitive tumor potentially reach the draining LN via dendritic cells (51). However, because in tumor-free satellite LNs, the in vitro T_{reg} suppressive activity appeared to be lower than in metastatic LNs, one may predict that it is in such satellite LNs that the detection of ongoing not yet fully inhibited antitumor immune response should be easier to isolate for further immunotherapeutic approaches (3).

Tumor-infiltrating $Foxp3^+CD4^+CD25^{high}$ T_{reg} were found, as expected, to operate in vitro through a cell contact mechanism. However, the degree of inhibition observed was almost correlated to the secretion of IL-10 by $CD4^+CD25^{high}$ and also $CD4^+CD25^-$ T cells in our in vitro assay. In addition, anti-IL-10 or anti-TGF-β_{1-3} Abs could revert this inhibition indicating that part of the mechanism may also be cytokine-dependent. However, the drastic effect of anti-TGF-β_{1-3} Ab contrasts with the fact that $CD4^+CD25^{high}$ T cells were rarely found to secrete TGF-β_1, the only TGF-β isoform detected by our ELISA. Because real-time PCR analysis did not allow us to detect any expression of either TGF-β_2 or TGF-β_3 isoforms by $CD4^+CD25^{high}$ T cells (data not shown), this suggests that the anti-TGF-β_{1-3} blocking Ab may exert its effect through cell surface-bound TGF-β1, which was recently implicated in the mediation of cell-contact-dependent immunosuppression by $CD4^+CD25^{high}$ T cells (52). It is of course difficult to extrapolate in vivo the mechanisms of suppression suggested by in vitro experiments. This is well illustrated in the mice model were the mode of action of T_{reg} in vivo remains unclear and is still largely debated (14). To explain the in vivo suppressive activity of T_{reg} at low $CD4^+CD25^{high}$:$CD4^+CD25^-$ ratio while needing direct cell contact, several groups (53–56) have shown that $CD4^+CD25^{high}$ T_{reg} may convey a suppressive activity to other $CD4^+$ T cells that was found to be contact independent and either IL-10 (55) or TGF-β dependent (56), a phenomenon known as infectious tolerance (57). Thus, it is possible that the cytokine-dependent suppressive activity observed in our study may reflect

the presence of such de novo-generated suppressive T cells. In contrast, it has been well documented that T_{reg} with cytokine-dependent suppressive activity could be induced from mature $CD4^+$ T cells under defined conditions of Ag stimulation in the presence of immunosuppressive drugs or cytokines (28, 58, 59). Our data suggest that Tr1/Th3-like T cells (22, 23) secreting both IL-10 and TGF-β could be detected in melanoma metastatic LNs among $CD4^+CD25^-$ T cells. Because melanoma cells have been described to secrete either IL-10 (60) or TGF-β (61), it is possible that the tumor cells themselves participate locally to the induction of such $CD4^+CD25^-$ suppressor T cells. In addition, it is noteworthy that TGF-β has been shown to promote the expansion of $CD4^+CD25^{high}$ T_{reg} (62) and that blockade of TGF-β signaling has been recently implicated in the immune-mediated eradication of tumors (63). This suggests that melanoma cells could also use TGF-β secretion to induce the local expansion of $Foxp3^+CD4^+CD25^{high}$ T_{reg} observed in our study. Taken together, the increased proportion of infiltrating $Foxp3^+CD4^+CD25^{high}$ T_{reg} as well as the suppressive activity of infiltrating $CD4^+CD25^-$ T cells support the idea that the local suppressive network is likely to be complex in metastatic melanoma and that distinct subtypes of suppressive T cells may be involved.

It has been recently shown in a murine model of *Leishmania major* infection that $CD4^+CD25^{high}$ T_{reg} control the persistence of the parasite by suppressing, through both IL-10-dependent and IL-10-independent mechanisms, the ability of $CD4^+CD25^-$ effector T cells to eliminate the parasite (64). In addition, the maintenance of strong resistance to reinfection, known as concomitant immunity, is also controlled by such $CD4^+CD25^{high}$ T_{reg}, suggesting that the recirculating pool of memory cells is maintained while effector T cells are suppressed locally (64). This situation strikingly parallels some features of tumor immunology where concomitant immunity is a well-known phenomenon (65) and where local immunosuppression appears to be important (10). Further studies on melanoma patients are needed to determine whether infiltrating $CD4^+CD25^{high}$ T_{reg}, as in the *L. major* model, effectively suppress in vivo local effector immune responses. To this respect, immunotherapeutic strategies aimed to counteract in vivo the action of T_{reg} might provide important answers to this hypothesis and would have crucial impacts on the designing of efficient vaccination protocols for the treatment of melanoma patients.

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