Cancer Cells Regulate Lymphocyte Recruitment and Leukocyte-Endothelium Interactions in the Tumor-Draining Lymph Node

Virginie Carrière,1 Renaud Colisson,1 Carine Jiguet-Jiglaire,1 Elisabeth Bellard,1 Gérard Bouché,1 Talal Al Saati,1 François Amalric,1 Jean-Philippe Girard,1 and Christine M'Rini1,2

1Laboratoire de Biologie Vasculaire, Equipe labellisée “La Ligue 2003”, Institut de Pharmacologie et de Biologie Structurale, CNRS UMR 5089; Plateau Technique d’Histopathologie Expérimentale IFR30, CHU Purpan; and 2Laboratoire de Physiologie, Faculté de Médecine de Rangueil, Toulouse, France

Abstract

The physiologic function of the secondary lymphoid organs to recruit large numbers of naïve lymphocytes increases the probability that antigens encounter their rare, sometimes unique, specific T lymphocytes and initiate a specific immune response. In peripheral lymph nodes (LNs), this recruitment is a multistep process, initiated predominantly within the high endothelial venules (HEVs), beginning with rolling and chemokine-dependent firm adhesion of the lymphocytes on the venular endothelium surface. We report here that, in C57BL/6 mice, the recruitment of naïve lymphocytes is impaired in LNs draining a B16 melanoma tumor. Intravital microscopy analysis of the tumor-draining LNs revealed that this effect is associated with an important deficit in lymphocyte adhesion in the HEVs and a progressive decrease in the expression of the LN chemokine CCL21. In parallel with these effects, the tumor up-regulated, essentially through a P-selectin–dependent mechanism, the rolling and sticking of circulating polymorphonuclear cells within the LN low-order venules where few rolling and sticking events are usually observed. These effects of the tumor were independent of the presence of metastasis into the LN and occurred as long as the tumor developed. Together, these results indicate that the tumor proximity disturbs the LN physiology by modifying the molecular, spatial, and cellular rules that usually control leukocyte-endothelium interactions into the peripheral LNs. In addition, they emphasize a new role for the low-order venules of the peripheral LNs, which compared with the HEVs, seem to be the preferential port of entry for cells linked to inflammatory processes. (Cancer Res 2005; 65(24): 11639-48)

Introduction

The rapid initiation of any T cell immune response depends on the efficient encounter between antigen-specific T cells and their cognate antigen. Secondary lymphoid organs are strategically positioned throughout the body to assume this task and more particularly to allow the encounter of foreign antigens with the largest possible number of naïve T lymphocytes, among which, few (sometimes only one) are able to mount a specific immune response. Among the secondary lymphoid organs, the peripheral lymph nodes (LNs) recruit naïve lymphocytes from the blood in a rhythm such that the entire circulating lymphocyte pool will visit at least one LN in 24 hours. Lymphocytes enter the LNs predominantly via the high endothelial venules (HEVs), the first venules emitted from the LN capillary network (1, 2) and, to a much lesser extent, via the low order venules (LOVs) that incrementally follow the HEVs (3). This is a highly regulated process involving different adhesion molecules and specific chemokines acting in a subtle combination set up to precisely select which blood cells will or will not enter the LN (4, 5).

In situ and in vivo observations of LN microcirculation by conventional (6), and more recently, by two-photon intravital microscopy (7), have over the last few years, contributed greatly to the characterization of the timing, spatial and cellular preferences, and the molecular mechanisms that mediate lymphocyte recruitment into the LNs. It begins by tethering and rolling events that slow down the cells circulating at very high speed in the blood. This first step concerns the majority of leukocytes without discrimination between monocytes, granulocytes, and lymphocytes (8). It is induced by the binding of L-selectin (CD62 L) on the leukocyte surface (2) to endothelial glycoproteins that are collectively named the peripheral node addressins (PNAd; ref. 9), are recognized by the monoconal antibody (mAb) MECA-79 (10), and are the molecular hallmark of HEV endothelial cells (11). The rolling cells, which now circulate much more slowly and in closer proximity to the endothelium wall, can therefore be attracted by chemokines, particularly CCL21 (12), which is constitutively (13) expressed along the LN venules (14). The presence or absence of receptors for CCL21 on the leukocyte surface determines which cells will or will not undergo the second step. Naïve T and B lymphocytes (as well as subsets of memory lymphocytes and dendritic cells) expressCCR7, a receptor for this chemokine (15). CCL21 binding induces the functional and conformational activation of the lymphocyte B2 integrins, particularly LFA-1 (CD11a/CD18; ref. 16) that, consequently, can bind endothelial intercellular adhesion molecule (ICAM)-1 and ICAM-2 molecules and initiates the third step, i.e., the firm adhesion or sticking of the lymphocyte to the venular endothelium (8). These first three steps are particularly critical and determine the entire homing process. From this point onwards, the bound lymphocyte cannot reenter the circulation and begins to transmigrate through the endothelium. Following gradients of the chemokines CCL21, CCL19, and CXCL13, it reaches the T zone or the LN follicles depending on its cell type (T or B lymphocyte; ref. 17).

After years of controversy, recent evidence shows that the immune system is involved in the defense of the host against cancer and is capable of recognizing and eliminating primary tumors (for a historical review, see ref. 18). To date, the identification of specific
tumor antigens (19), the positive correlation between the presence of lymphocytes within the tumor and increased patient survival (20), the increased frequency of malignancies in patients with severe immune deficiencies (21), the necessity for intact lymphocyte antigen receptor rearrangement (22), and effector functions (23), have all been conducive to the idea that the presence of a tumor triggers a specific and effective immune response. However, the role of the immune system in this process seems to be complex, progressively leading to the selection of tumor cells of reduced immunogenicity and thus high malignancy. To escape from the immune response, the tumor sets up several mechanisms of as yet undetermined importance (for reviews, see refs. 24, 25). Most of these mechanisms take place during the effector phase of the antitumor immune response, and it is generally considered that the priming phase, during which tumor antigens are presented to the naive lymphocyte pool, is unaffected. However, this has not been extensively studied.

In this study, we used conventional intravital microscopy to examine whether the very early preparatory phase of the immune response, i.e., the recruitment of large numbers of naive lymphocytes into LNs, is affected by the development of a tumor in the node's vicinity. We showed that the proximity of a tumor induced a defect in lymphocyte adhesion and homing through the HEVs in parallel with a progressive decrease in expression of the CCL21 protein by the LN tissue. We found that the tumor induced an up-regulation of the rolling and sticking of polymorphonuclear cells (PMN) within the LOVs, which is dependent on P- and L-selectins and PSGL-1, and which leads to their recruitment into the LN. Finally, we showed that these tumor-induced effects were independent of the presence of metastasis into the LN and occurred as long as the tumor developed and the animal survived.

Materials and Methods

Animals. Five- to seven-week-old female C57BL/6 mice were purchased from the Iffa-Credo Laboratory (L’Isle sur Sorgue, France). Mice were housed and bred in a specific pathogen–free animal facility. Experiments were conducted in accordance with French procedural guidelines for animal handling.

Reagents. The mAb directed against murine PSGL-1 (mAb 4R10 rat IgG) was kindly provided by Dr. Vestweber (Max-Planck-Institute of Molecular Biomedicine, Munster, Germany). Polyclonal antibody to CCL21 and recombiant CCL21 were purchased from R&D Systems (Lille, France). All other mAbs and human recombinant tumor necrosis factor-α (TNF-α) were from Becton Dickinson Pharmingen (Le Pont de Claix, France). Fluorescein dextran (with molecular weights of 10,000 and 150,000; 10 and 150 KDa FITC-dextran), rhodamine 6G, calcine AM, chloromethylfluorescein diacetate and NeutrAvidin-labeled Fluospheres were purchased from Molecular Probes (Invitrogen, Cergy Pontoise, France).

Tumor cell lines and implantation. Two variants of low (B16F1) and high (B16F10) metastatic potential of the spontaneously arising murine melanoma line B16 derived from C57BL/6 mice were used and maintained as described (26). B16 cells (10⁶) were inoculated in 0.1 mL of PBS s.c. upstream of the subiliac LN in the left flank of the mice. The mice were sacrificed at different days after the implantation (days 1, 5, 10, 15, and 20) or for humane reasons when tumors reached 20 mm in their largest dimension or when ulceration and/or bleeding developed.

Intravital microscopy of subiliac lymph node. Unchallenged and tumor-bearing mice were anesthetized by i.p. injection of 1 mg/mL of xylazine and 5 mg/mL of ketamine. The left subiliac LN was prepared for intravital microscopy as previously described (3) and the mouse was then transferred to a customized intravital video microscopy setup (INM 100; Leica Microsystems SA, Rueil-Malmaison, France) equipped with water immersion objectives (HCX APO; Leica Microsystems). Fluorescent events in the LN microcirculation were visualized and recorded by a silicon intensified target camera (Hamamatsu Photonics, Massy, France) and stored on DVC camera tape devices (DSR-11 Sony, IEC-ASV, Toulouse, France).

For visualization of naive lymphocyte interactions with LN vascular endothelium, naive lymphocytes obtained from single T and B cell suspensions of a control C57BL/6 pool of subiliac, axillary, and brachial LNs were fluorescently labeled by calcine AM (0.25 μM/L for 5 minutes at 37°C) and injected i.v. (27). For visualization of endogenous leukocyte interactions, 1 μg/mL of rhodamine 6G (5) was directly injected into the mouse circulation. In some experiments, 30 μg mAb Mel-14 and/or 30 μg mAb 10E9, mAb RB40.34, mAb 4R10, and/or 100 μg MECA-79 were injected i.v., and leukocyte behavior was recorded 15 minutes thereafter. The luminal surface area of the LN microvessels and cell behavior in LN venules were calculated or assessed as previously described (2). The rolling fraction was determined as the percentage of cells that rolled along the vascular lining in the total flux of cells per venule. The sticking fraction was determined as the percentage in rolling cells of cells firmly adherent for at least 30 seconds. In experiments using rhodamine 6G-labeled leukocytes, the number of firmly adherent cells per luminal surface area was calculated for each venule (number of cells stuck per unit of surface area).

For mapping of antigen densities and distribution in the LN microvessels, NeutrAvidin-yellow green (Ex/Em, 505/515 nm) or -red (Ex/Em, 580/605 nm) fluorescent microspheres (1 μm diameter) were labeled with 10 μg/mL of either the biotinylated isotype-matched control mAbs or the biotinylated MECA-79 or anti-L-selectin or anti-P-selectin mAbs, injected i.v. and enumerated in the LN venular tree as previously described (3). In some experiments, 500 ng TNF-α in 100 μL PBS were s.c. injected where tumor cells are usually implanted.

Homing experiments. A single-cell suspension of naive B and T lymphocytes was prepared from a pool of peripheral LNs including the brachial, axillary, and subiliac LNs from control C57BL/6 mice, labeled with 5 μM/L 5-chloromethylfluorescein diacetate and injected (2 x 10⁶ cells in 300 μL PBS) i.v. into recipient mice as described (28). After 24 hours, single-cell suspensions from unchallenged and tumor-draining subiliac LNs were prepared and the percentage of fluorescent cells was assessed by flow cytometry.

Immunohistochemistry. Cryostat-cut serial LN sections (5-8 μm) were mounted on slides, fixed for 10 minutes in acetone, air-dried overnight, and stored desiccated at −80°C. Immediately before use, sections were hydrated in PBS supplemented with 0.3% bovine serum albumin. Endogenous peroxidase and biotin activities were blocked with specific reagents. Polyclonal goat antibody anti-mouse CCL21 (1:1,000) was applied followed by rabbit anti-goat secondary antibody (1:1,000; DakoCytomation, Trappes, France). Staining was revealed by application of EnVision horseradish peroxidase system and 3,3’-diaminobenzidine. Sections were counterstained with hematoxylin and mounted with mounting media.

Western blot analysis. Unchallenged or tumor-draining subiliac LN was homogenized in ice-cold lysis buffer containing protease inhibitor cocktail (Complete, Roche, Meylan, France) according to a previously published procedure (29). Whole LN homogenates were loaded and separated by denaturing polyacrylamide (12%) gel electrophoresis. Recombinant CCL21 (5 ng) was loaded in parallel to LN extracts. Proteins were transferred onto a nitrocellulose membrane and the membrane was probed with the polyclonal goat anti-mouse CCL21 at a dilution of 1:1,000, followed by a horseradish peroxidase–conjugated rabbit anti-goat secondary antibody at 1:10,000 (DakoCytomation). Proteins were visualized with the enhanced chemiluminescence system (Amersham Biosciences, Orsay, France).

Statistical analysis. For statistical comparison of paired samples, a two-tailed Student’s t test was used. Multiple comparisons were done using the Kruskal-Wallis test with the Bonferroni correction. Differences were considered statistically significant when P < 0.05 (** or P < 0.01 (***)). Data are presented as mean ± SE.

Results

Establishment of B16F1 and B16F10 melanoma in C57BL/6 mice. The injection of 10⁷ murine melanoma B16 cells s.c. into the
flank of syngeneic C57BL/6 mice lead to the development of malignant tumors that killed the mice between the 30th and the 45th day after implantation. The growths of low (B16F1) and high (B16F10) metastatic potential tumors were identical (Fig. 1A). After B16F10 tumor cell implantation, metastases became apparent in the homolateral subiliac LN between the 10th and the 15th day (Fig. 1B). Because we observed that injection of fluorescent dextran (150 or 10 kDa) into the usual site of the tumor cell implantation was followed 10 minutes afterwards by the appearance of fluorescent molecules in the subiliac homolateral LN (Fig. 1C), we referred to this node as the tumor-draining LN.

Homing and sticking of naïve lymphocytes is impaired in the tumor-draining lymph node. To study whether the physiologic function of LNs to recruit large numbers of naïve lymphocytes is maintained when a tumor is developing in its vicinity, exogenously prepared fluorescent naïve lymphocytes were injected i.v. into unchallenged and tumor-bearing mice and their presence into the inguinal draining-LNs was investigated by flow cytometry 24 hours afterwards. We found that the number of fluorescent naïve lymphocytes recovered into the tumor-draining LNs was partially and significantly decreased in comparison to the number of such cells that homed during the same period of time into LN of unchallenged mice or into a LN draining a s.c. area into which 100 µL of phosphate buffer medium containing 1% FCS (PBS-draining LN; Fig. 2A) had been injected.

To investigate the mechanisms of this defect, an intravital microscopy examination of the tumor-draining LN and its microcirculation was done. This examination showed that, as for the unchallenged node (2), the venular tree of the tumor-draining LNs consists of up to five branching orders that can be separated in LOVs and HEVs. The LOVs comprise the large collecting venule in the hilus (order I) and upstream branches in the medulla (order II and certain order III venules). The HEVs are higher order branches (most order III and all order IV and V venules) that are directly connected to the LN capillaries and flow towards the LN subcortex and paracortex (Fig. 2B). In unchallenged LN, intravital microscopy observations of exogenously prepared fluorescent naïve lymphocytes injected i.v. into the recipient mice have shown that rolling and sticking are mainly supported by the HEVs but can also occur in the LOVs (refs. 2, 8; Fig. 2C and D). In the LOVs of the B16F10 tumor-draining LNs, naïve lymphocytes rolled a little bit more (although this is not statistically significant), and stuck identically compared with the LOVs of unchallenged LN (Fig. 2C and D). In the HEVs of the B16F10 tumor-draining LNs, naïve lymphocytes displayed as many rolling events as in unchallenged vessels (Fig. 2C) but were not able to stick as frequently, leading to a severe diminution of the sticking fractions inside order IV and V HEVs of

**Figure 1.** B16F1 and B16F10 melanoma model in C57BL/6 mice. A, B16F1 and B16F10 tumor growth. Tumor volume was measured daily along two perpendicular axes (x and y) and estimated as being \( \pi/6 \times x \times y^2 \). Points, mean of 10 separate experiments. B, histologic examination (magnification, ×400) of homolateral subiliac LN sections (7 µm thick) show the presence of metastasis (Mts) 15 days after the s.c. implantation of B16F10 melanoma cells. C, fluorescence images of homolateral and contralateral subiliac LN sections (7 µm thick) 10 minutes after s.c. injection of fluorescein-labeled dextran (FITC-dextran, 150 or 10 kDa) into the left flank of an unchallenged C57BL/6 mouse. FITC-dextran (150 kDa) filled the subcapsular and medullary sinuses of the homolateral LN with minimal penetration into the cortex. FITC-dextran (10 kDa) entered the LN cortex and was distributed through both the homolateral and contralateral LNs.
the tumor-draining LNs (Fig. 2D). This impairment in the capacity of naïve lymphocytes to firmly adhere to the HEVs of a LN draining a B16F10 tumor was observed as early as 24 hours after the tumor cell implantation, and lasted as long as the tumor developed and the host mouse survived (Fig. 2D).

Figure 2. The proximity of a B16 melanoma impairs the sticking of naïve lymphocytes inside the HEVs of the tumor-draining subiliac LNs. Homing of chloromethylfluorescein diacetate–labeled naïve lymphocytes in unchallenged LN, or in LN draining a s.c. area into which 100 μL of phosphate buffer medium containing 1% FCS had been injected (PBS-draining LN) or in tumor-draining LN. At least four recipient mice were tested for each experimental group. For the tumor-draining experimental group, experiments were done at different days following the injection of the B16F1 tumor cells and results were pooled. B, intravital micrograph and its sketch show a typical subiliac LN at low magnification (×50). LN venous blood drains into an extralymphoid side branch of the superficial epigastric vein via the LOVs, which comprise a large collecting venule (order I) and order II and some order III venules, and upstream, via the HEVs which comprise most order III and all order IV and V venules. C, rolling fractions and sticking fractions (D) of calcein-labeled naïve lymphocytes in the venular tree of subiliac unchallenged wild-type LNs or tumor-draining LNs 1, 5, 10, and 15 day(s) after implantation of B16F10 cells. Columns, mean of 10 experiments (4-10 venules each); bars, ± SE; *, P < 0.05; **, P < 0.01 versus unchallenged LN. E, sticking fractions of calcein-labeled lymphocytes in the HEVs (order IV and V venules) of unchallenged or 1% FCS PBS, B16F1, or B16F10 tumor-draining LNs. Experiments were done on different days following the injection of the 1% FCS PBS or the B16 tumor cells and the results were pooled according to the LN draining-type and the venular order. Columns, mean of a minimum of five experiments (4-10 venules each); bars, ± SE; *, P < 0.05; **, P < 0.01 versus unchallenged and 1% FCS PBS-draining LNs.
one. Its proximity provoked a striking decrease in lymphocyte sticking within the B16F1 tumor-draining LN HEVs (Fig. 2E) but had no effect on rolling and sticking cell behavior inside the LOVs (data not shown) confirming that the decreased capacity of lymphocytes to firmly adhere inside the HEVs of a B16 tumor-draining LN was independent of the presence or absence of metastasis into the lymphoid tissue.

Tumor induces a progressive decrease of CCL21 expression in the draining lymph nodes. In an effort to identify the molecular mechanism of the impaired ability of naïve lymphocytes to stick inside the tumor-draining LN HEVs, we analyzed the potential role of CCL21. We designed immunohistochemical studies for the analysis of CCL21 expression in frozen sections from both unchallenged and tumor-draining LNs (Fig. 3A). In agreement with previous reports (30), the strongest CCL21 staining in unchallenged LNs was found on the MECA-79+ HEV endothelial cells and in disseminated cells of the LN paracortex and subcortex (Fig. 3A). In tumor-draining LNs, CCL21 staining was still present on the endothelial and stromal cells. However, it seemed to be weaker than in the unchallenged LNs. We also examined CCL19 that we found also still present in the tumor-draining LN sections. However, the faint staining did not allow a clear evaluation of any possible difference between the unchallenged and tumor-draining LNs (data not shown). Immunohistochemical analysis of ICAM-1 and ICAM-2 expression and of B220+ B lymphocytes, CD4+ and CD8+ T lymphocytes, interdigitating dendritic cells (DEC-205+ cells), and follicular dendritic cells didn’t provide evidence for any significant difference or major topographical changes between unchallenged and tumor-draining LN sections, except for the emergence of follicular hyperplasia with a lightening and an unchallenged and tumor-draining LN sections, showed the presence of Gr-1+ cells in the medulla, upper cortex, and capsule of the LN from day 5 after tumor implantation (Fig. 4D). F4/80, CD11b, CD11c, and B220 were not found colocalizing with the Gr-1+ staining, suggesting that the Gr-1+ cells were exclusively PMNs.

The proximal tumor induces the expression of P-selectin at the luminal surface of the draining lymph node venular tree. The increased rolling of PMNs observed in the LOVs of tumor-draining LNs suggested an inflammatory reaction. The multistep paradigm for leukocyte recruitment during inflammation involves an initial rolling step mediated by L-selectin (31) and/or the P- and E-selectins that are known to be induced at the surface of the endothelium during the inflammatory response (32, 33). To examine whether these molecules were induced in the tumor-draining LN venular tree, semiquantitative analysis of luminal surface molecules was done by intravital microscopy using fluorescent beads as previously described (3). In unchallenged LNs, anti-P-selectin–coated beads, when injected i.v., bound sparsely throughout the venular tree (Fig. 5A and B), likely reflecting the transient induction of P-selectin at the surface of LN venules observed after surgical exposition of the LN for intravital examination (34). In tumor-draining LNs, the number of anti-P-selectin–coated beads that accumulated in the venular tree was greater than that seen in the unchallenged LNs, although not as great as the number of beads bound in the venular tree of a LN challenged by TNF-α (Fig. 5A and B). P-selecting binding sites were ubiquitously distributed throughout the LN venules including the LOVs and the HEVs. Anti-E-selectin–coated beads did not accumulate significantly in the venular tree of the tumor-draining LN, although they bound in high numbers in the venular tree of LN challenged by TNF-α (Fig. 5A and B). MECA-79-coated beads accumulated preferentially in the HEVs, as was the case in unchallenged LN venules (Fig. 5A and B), corroborating our histochemical studies of LN sections, which showed that the tumor proximity does not affect PNA d expression in the LN venules.

The increased rolling of polymorphonuclear cells in the low order venules of the tumor-draining lymph nodes is L-selectin-, P-selectin-, and PSGL-1–dependent. To determine whether the induction of P-selectin is at the origin of the increased rolling of PMNs observed in the LOVs of the tumor-draining LNs, we studied the effects on this rolling, of specific anti-L-, P-, and E-selectin mAbs injected i.v. to the mouse, alone or in combination (Fig. 6). Injection of the anti-L-selectin mAb significantly attenuated the tumor-induced increased PMN rolling in the LOVs, without inhibiting it entirely. In the order I venules, the inhibition induced by injection of this mAb was ~50%, but it increased in parallel with increasing venular order, leading to an inhibition of almost 80% in the order IV and V HEVs. Injection of the specific anti-P-selectin mAb inhibited rolling in order I venules by >90% but had no significant effect in the HEVs. Concomitant injection of both
anti-L-selectin and anti-P-selectin mAbs did not significantly modify the inhibitory effect induced by one or the other mAb used alone. Injection of a specific anti-E-selectin mAb had no effect on leukocyte rolling in the LOVs (Fig. 6), nor did it decrease the residual rolling of leukocytes observed after anti-P-selectin mAb treatment (data not shown). Taken together, these results suggested that the principal mediators of the increased blood cell rolling observed in the tumor-draining LOVs was endothelial P-selectin with a lower but still significant contribution of L-selectin.

Next, we examined the implication of PSGL-1 either as a direct ligand for endothelial P-selectin (35), or as an L-selectin ligand that induces second capture events and thus an amplification of leukocyte rolling (36). Blockade of PSGL-1 inhibited leukocyte rolling by >80% in the order I venules whereas its effect decreased with increasing venular order (Fig. 6). Injection of anti-PSGL-1 mAb in combination with the anti-L-selectin and P-selectin mAbs provoked an almost complete inhibition of blood cell rolling in the LOVs, resulting in residual rolling of <10%. The injection of pairs of these mAbs instead of all three simultaneously showed that in the order I venules, PSGL-1 was the predominant counter-receptor for endothelial P-selectin, whereas in the order II venules, it shared this role with L-selectin. The involvement of these molecules in the increased blood cell rolling observed in the LOVs of tumor-draining LN remained unchanged throughout the time course of tumor development (data not shown).

Previous studies have shown that, in node venules, rolling is mediated predominantly by MECA-79-positive PNAds but also, particularly in the LOVs, by MECA-79-insensitive ligands (3). It has also been shown that both ligands can be induced in inflammation (11, 37). To test the implication of the MECA-79-dependent or -independent ligands in our observations, injection of MECA-79 mAb was done alone or in combination with the anti-P-selectin mAb.
and anti-PSGL-1 mAbs (Fig. 6). Surprisingly, and in contrast with the usual role of MECA-79 in the HEVs of unchallenged LNs (3, 6), the MECA-79 mAb was unable to abolish or reduce leukocyte rolling inside any of the tumor-draining LN venules. However, when the MECA-79 mAb was injected in combination with the anti-P-selectin and PSGL-1 mAbs (that alone have no effect on the rolling observed inside HEVs, as discussed above), rolling was markedly reduced, to a residual value of ~20%. Taken together, these results suggested that in tumor-draining LN HEVs, L-selectin remained the principal mediator of blood cell rolling, indiscriminately using two types of endothelial ligands, the PNAds and/or the tumor-induced P-selectin. There was no evidence to suggest that MECA-79-insensitive ligands were induced in our model because the residual rolling observed after injection of a combination of anti-MECA-79 and anti-P-selectin mAbs was approximately the same with the residual rolling observed after anti-L-selectin mAb treatment. Obviously, there was another molecular pathway that accounted for 10% to 20% of rolling inside the tumor-draining LOVs and HEVs that was not mediated by any of the selectins.

**Figure 4.** The proximity of a B16 melanoma triggers increased rolling and sticking events spatially restricted to the LOVs in tumor-draining LNs. A, rolling fractions of rhodamine 6G-labeled circulating leukocytes were assessed in each venular order of peripheral LNs from unchallenged and tumor-draining LNs 1, 5, 10, and 15 day(s) after implantation of B16F10 cells. Columns, mean of 10 experiments (4-10 venules each); bars, ± SE. Experiments were done at different days following the injection of 100 μl of phosphate buffer medium containing 1% FCS (PBS-draining LN) or the B16 tumor cell implantation and results were pooled according to the LN draining-type and the venular order. *, P < 0.05; **, P < 0.01 versus unchallenged LN. B, leukocyte sticking in order I and II LOVs of B16F1 and B16F10 tumor-draining LNs and of LNs draining a s.c. area in which 1% FCS PBS-draining LN was injected. Columns, mean of at least three experiments (4-10 venules each); bars, ± SE. Experiments were done at different days following the injection of 1% FCS PBS or the B16 tumor cell implantation and results were pooled according to the LN draining-type and the venular order. *, P < 0.05; **, P < 0.01 versus 1% FCS PBS-draining LN. C, this intravital micrograph (magnification, × 400) shows rhodamine 6G-labeled leukocytes stuck on the surface of an order I venule of a tumor-draining LN at day 15. The nuclear staining pattern of irregular shape was characteristic of PMNs (arrowheads). D, histologic examination (magnification, × 100) of a tumor-draining LN section (5 μm thick) at day 5 after B16F1 tumor cell implantation. The section was stained in brown for Gr-1 (Ly-6G) epitopes, mostly expressed by PMNs.

**Discussion**

The rapid initiation of any immune response depends on the efficient encounter between antigen-specific T cells and their cognate antigen. Secondary lymphoid organs, which include the peripheral LNs, are strategically positioned throughout the body to assume this task. To escape from the immune response, the tumor sets up several mechanisms (25), the majority of which take place during the effector phase of the antitumor immune response. It is generally considered that the priming phase during which tumor antigens are presented to the naïve lymphocyte pool in secondary lymphoid organs, is unaffected. However, this has not been extensively studied and little data is available on LN ultrastructure and function in the proximity of a genuine tumor.

Using a mouse tumor model, we report here that the spatial, molecular, and cellular rules that usually control the entry of naïve lymphocytes into the LN are significantly altered by the presence of a tumor developing in its proximity.

We found that the proximity of a tumor induces a progressive down-regulation of the LN CCL21 content as the tumor develops. Interestingly, a similar down-regulation of CCL21 has recently been shown to occur in human LNs bearing malignant metastatic tumor cells (38). In our study, the simple presence of the tumor in the periphery of the LN was sufficient to induce the down-regulation of CCL21 even in the absence of detectable tumor cells in the LN tissue. It is known that this chemokine is constitutively expressed in the node tissue and can be induced during chronic inflammation and autoimmune reactions in other tissues (39, 40). Our observations report for the first time that CCL21 can also be subjected to down-regulation. We showed that this down-regulation is associated with a defect of naïve lymphocyte sticking inside the tumor-draining LN HEVs, and to a lesser extent, to a defect in lymphocyte homing into the node tissue. CCL19, the other node constitutive chemokine, and ICAM-1 and ICAM-2, which are also implicated in the firm adhesion of lymphocytes in the LN HEVs, are not significantly modified in the tumor-draining LN, suggesting that the defect observed in the adhesion and recruitment of naïve lymphocytes has to be linked predominantly to the CCL21 down-regulation induced by the tumor proximity. Interestingly, this down-regulation did not seem to have any effect on cell positioning inside the node tissue because the ultrastructure of the tumor-draining LN was not macroscopically altered. Whether the other functions of CCL21 are affected (such as the recruitment of dendritic cells from the tumor area into the LN) will have to be examined further.

We also showed that tumor development is followed by a slight increase in the number of circulating PMNs (<5%; data not shown).
and the emergence of PMNs within the draining LN, suggesting that the tumor induced an inflammatory or pseudo-inflammatory reaction in its periphery. Although it cannot be excluded that PMNs homed into the draining LN from the tumor area via the lymph, the increased rolling and sticking events observed in the LN venules strongly suggest that at least a fraction of the PMNs have been directly recruited from the blood. This nonphysiologic recruitment of PMNs into the tumor-draining LN depends on the rolling step of the molecular mechanisms involving P-selectin (but not E-selectin), L-selectin, and PSGL-1. The stability in the molecular mechanisms involved differs from that observed in more common inflammatory reactions, where successive and overlapping phases occur, involving L-selectin and PSGL-1 present on leukocytes and P- and E-selectins expressed by activated endothelial cells (41, 42). In contrast to observations made in other inflammatory models (43), we showed that this tumor-induced

Figure 5. Fluorescent bead accumulation reveals an up-regulation of luminal P-selectin expression in the venular tree of B16 tumor-draining LNs. Binding of specific mAb-coated yellow green or Nile red fluorescent beads were (A) observed by intravital microscopy (intravital micrographs obtained with a 10× objective) and (B) semiquantitatively evaluated in the venular tree of unchallenged LNs, B16F1 tumor-draining LNs, and LNs draining a s.c. area into which 500 ng TNFα in 100 μL PBS (TNFα-draining LN) had been injected. Beads were coated with either the anti-MECA-79 mAb, the anti-P-selectin mAb RB40.34, or the anti-E-selectin mAb 10E9.6. The accumulation of specific beads was calculated as described in Materials and Methods, and is reported according to the venular order, except for the TNFα-draining LN for which the mean reported was obtained from all the venules whatever their order. Columns, mean of at least three experiments (4-10 venules each); bars, ± SE. All specific mAb-coated beads bound significantly more than the isotype control beads, except for the MECA-79-coated beads in order I venules, and the anti-E-selectin-coated beads in unchallenged LN.
References


9. Berg EL, Robinson MK, Warnock RA, Butcher EC. The process restricts the sticking of PMNs to within a precise venular area, the LOVs that have been previously shown to be distinct from HEVs in terms of phenotype and function (3). Our results suggest that HEVs and LOVs could be used by distinct blood cell subsets as selective ports of entry into the LN, depending on the physiologic or pathologic context [for the HEVs, naïve lymphocytes under unchallenged situations and CXCR3+ monocytes (44) and elicited monocytes (45) under specific inflammatory situations; for the LOVs, PMNs during the inflammation or pseudo-inflammation process, as seen in our tumor-induced model]. The preference of the LOVs for PMNs may be explained by recent observations reporting that PMN cell counter-receptors for P-selectin have a much greater affinity for this selectin than those of the monocytes and lymphocytes (46), and that amplification of blood cell rolling through L-selectin-mediated second capture events is also known to preferentially involve PMNs (47). The preference of PMNs for LOVs could be explained by the necessity for the PMNs to have a prolonged rolling contact time with the endothelium to promote chemokine activation and integrin-mediated arrest (48). Thus, in our model, P-selectin molecules in the LOVs would relay the P-selectin and MEGA-79 epitopes expressed on the HEVs to give the rolling PMNs enough time to be activated and finally to firmly adhere to the surface of LOVs. Specific chemokine(s) for PMNs appearing uniquely on the surface of the LOVs could also explain the restriction of PMN sticking to these venules.

Our results suggest that the peripheral tumor may exert a “remote control” over the molecular and cellular function of the LN by projecting its local chemokine and/or cytokine profile to the draining LN, probably through interstitial fluid transported to the LN venules by the afferent lymph vessels and specialized conduits and channels (49). In agreement with this possibility, we found that low molecular weight dextran (similar to chemokines) that was injected into the same location as the tumor was drained extremely quickly to the adjacent inguinal LN. Interestingly, we also retrieved fluorescent low molecular weight dextran in the brachial, axillary, and contralateral inguinal LNs a few minutes after the injection (Fig. 1F), suggesting that local discharge of molecules by the tumor and its neighborhood may also have consequences at higher distances than initially considered.

Cancer is a progressive disease, occurring in a series of well-defined steps, typically arising as a consequence of activating or deactivating mutations in proliferating cells. It is now evident that single mutagenic events alone are not sufficient for the induction of a malignant tumor. Additional events are necessary for tumor progression. Among these, it has been recently shown that inflammation (50) and inflammatory cells, such as macrophages and PMNs, can potentiate neoplastic progression via paracrine factors (51, 52). However, published results are contradictory, and in animal models, some authors assume that the PMNs are active in immunosurveillance against tumors (53), whereas others report that their presence may favor malignant growth and tumor progression (54). It has not been previously considered that this ambiguous role of the PMNs in cancer could take place in the draining LNs, either in addition to, or instead of, within the tumor itself. Therefore, our data showing that tumor development is followed by the emergence of PMNs within the draining LN, opens questions on the agonistic or antagonistic role that these cells may play within the draining LN, especially on the elicitation of the specific antitumor lymphocyte-mediated immune response.

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Figure 6. Increased PMN rolling in tumor-draining LNs is dependent on L-selectin, P-selectin, and PSGL-1. Rolling fractions of rhodamine 6G-labeled leukocytes were analyzed in B16F1 tumor-draining LNs before (none) and after treatment of animals with blocking mAbs used alone or in combination. Columns, mean from three to seven experiments of 4 to 10 vessels each; bars, ± SE. Experiments were done at different days following the injection of the tumor cells and the results were pooled according to the venular order. HEVs regroup order IV and V venules. *, P < 0.05; **, P < 0.01; ***, P < 0.001 versus rolling fractions before any mAb treatment.
Cancer Cells Regulate Lymphocyte Recruitment and Leukocyte-Endothelium Interactions in the Tumor-Draining Lymph Node

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