

Treatment with transcatheter arterial chemoembolization induces an increase of the L-selectin^{low} CXCR3⁺ CD8⁺ T cell subset in patients with hepatocellular carcinoma

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Background: The purpose of this study was to investigate the impact of treatment with transcatheter arterial chemoembolization on the expression of chemokine receptors on memory T cells around tumor sites in vivo in patients with hepatocellular carcinoma.

Methods: Blood samples from the hepatic artery and a peripheral vein were collected from 100 patients with hepatocellular carcinoma before and 4 weeks after treatment with transcatheter arterial chemoembolization. Mononuclear cells were isolated and examined for the expression of L-selectin (CD62L) and CXCR3 (CD183) on CD8⁺ T cells in patients with hepatocellular carcinoma during transcatheter arterial chemoembolization.

Results: Both the frequency and number of L-selectin^{low} CXCR3⁺ proinflammatory effector T cells in patients with hepatocellular carcinoma increased significantly following treatment versus pretreatment ($61.92\% \pm 8.69\%$ versus $24.45\% \pm 7.36\%$, $P < 0.05$, and 18.98 ± 2.33 e7/L versus 6.10 ± 1.21 e7/L, $P < 0.001$, respectively). There was no significant difference in its frequency whether in the hepatic artery or peripheral vein. Furthermore, the frequency of CD69⁺ T cells in patients with hepatocellular carcinoma increased from $2.53\% \pm 0.51\%$ in the artery and $2.38\% \pm 0.49\%$ in the vein to $3.80\% \pm 0.62\%$ and $4.48\% \pm 0.75\%$, respectively, after treatment (both $P < 0.05$).

Conclusion: Treatment with transcatheter arterial chemoembolization may lead to an increase in L-selectin^{low} CXCR3⁺ effector T cells in patients with hepatocellular carcinoma.

Keywords: hepatocellular carcinoma, transcatheter arterial chemoembolization, T cells, L-selectin, CXCR3

Introduction

Hepatocellular carcinoma is a major health problem worldwide and responsible for approximately one million deaths annually.¹⁻³ Recent advances in therapy have improved survival rates,⁴ but little is known about the underlying immune mechanism. T lymphocytes are believed to constitute the main body of effectors in active immune protection against cancer. Paradoxically, many patients with detectable tumor antigen-specific T cells in their peripheral blood lymphocytes still continue to progress, suggesting that the existence of peripheral tumor-specific CD8⁺ effector cells alone may be insufficient to mediate immunologic control of tumors. One possibility is that many of the activated T cells fail to home to tumor sites. Accessibility of T cells to tumor compartments requires specific interactions between lymphocyte cell surface receptors, selectins, and integrins on the vascular epithelium, as well as

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between lymphocyte-expressed CC or CXC chemokine receptors and soluble chemokines derived from host tissues near the tumor or from the tumor itself. Likewise, successful T cell-mediated antitumor immunotherapy may rely on expression of CXC chemokine receptors on lymphocytes. L-selectin (CD62L) is a crucial adhesion molecule that regulates both migration of leukocytes at sites of inflammation and recirculation of lymphocytes between blood and lymphoid tissues. Chemokine (C-X-C motif) receptor 3 (CXCR3) is upregulated on activated T cells and plays a critical role in recruiting effector and effector memory T cells to enter peripheral inflammation tissue. High levels of CXCR3 (CD183) on circulating and tumor-infiltrating CD8⁺ T cells have recently been implicated in effective control of advanced melanoma^{5,6} and achievement of viral control in both chronic hepatitis B and C.^{7,8} An increasing number of studies have demonstrated that L-selectin^{low} CXCR3⁺ proinflammatory effector T cells facilitate the tissue inflammatory reaction.^{9,10} To date, no study has characterized the role of CXC chemokine receptors on activated T cells in vivo when treating hepatocellular carcinoma with transcatheter arterial chemoembolization. Given that transcatheter arterial chemoembolization leads to necrosis of tumor tissue, we hypothesized that proinflammatory molecules released from necrotic cells would stimulate proinflammatory L-selectin^{low} CXCR3⁺ effector T cells and encourage the inflammatory reaction. Therefore, in the present study, we investigated the impact of transcatheter arterial chemoembolization on expression of chemokine receptors on memory T cells around tumor sites in patients with hepatocellular carcinoma.

Materials and methods

This study was carried out in accordance with the Declaration of Helsinki and was approved by the ethics committee of Capital Medical University. All patients provided their informed written consent.

Patient selection

Between January 2010 and December 2010, 100 patients with hepatocellular carcinoma (86 men, 14 women) aged 51 ± 12 (range 21–78) years with no indication for surgical resection were enrolled in the study. Ten healthy volunteers, aged 20–38 years, were recruited from Capital Medical University. Hepatocellular carcinoma was diagnosed by distinctive findings on ultrasonography, computed tomography, magnetic resonance imaging, and angiography, and serum levels of alpha-fetoprotein. After being shown the results of previous clinical studies of transcatheter arterial chemoembolization,

all 100 patients selected this therapeutic option on the basis of informed consent. All of the enrolled patients met the eligibility criteria for inclusion in the analysis, as described in the next paragraph.

Transcatheter arterial chemoembolization procedure

Initially, selective angiography was carried out in all patients. After identifying the tumor-feeding artery, transcatheter hepatic segmental arterial chemoembolization using anticancer drugs was performed comprising 1000 mg of 5-fluorouracil, 20 mg of hydroxycamptothecin cisplatin or 10–20 mg of mitomycin C, and then 5–20 mL ethiodized oil (lipiodol, André Guerbet, Aulnay-sous-bois, France), followed by injection of Gelfoam particles. The mixture of lipiodol and anticancer drugs was selectively infused into the hepatic vessels supplying tumor-bearing tissue until the lipiodol was thickly accumulated in the tumor tissue and tumor-bearing area. The arteries were then embolized using gelatin sponge particles. The volume of injected lipiodol in each patient was 2–30 mL according to tumor size, vascularity, and fluoroscopic findings. Transcatheter arterial chemoembolization treatment was repeated if there was any residual tumor detected after 4 weeks.

Blood collection and preparation of mononuclear cells

After identifying the tumor-feeding artery, 10 mL of blood from the hepatic artery and 10 mL of blood from a peripheral vein was collected from the same patients. Mononuclear cells were prepared from freshly drawn blood using standard Ficoll-Paque (Gibco, Grand Island, NY) density gradient centrifugation. The cells were washed twice with sterile phosphate-buffered saline and resuspended in complete RPMI 1640 medium (Gibco) containing 2 mmol/L L-glutamine (Gibco), 10% (vol/vol) heat-inactivated fetal calf serum (Gibco), 100 IU/mL of penicillin, and 100 µg/mL of streptomycin (Gibco). The cell concentration in the final suspension was $1.0\text{--}2.0 \times 10^6$ cells/mL.

Multiparametric phenotypic characterization of T subsets

Assays were performed on freshly isolated mononuclear cells. For phenotypic analysis, one million mononuclear cells were stained with freshly prepared antibodies, ie, fluorescently labeled CD3-PeCy7, CD8-fluorescein isothiocyanate, L-selectin phycoerythrin, and CXCR3-PeCy5 for 30 minutes

at 4°C in the dark. Staining was performed in a final volume of 100 µL. All antibodies were titrated beforehand to achieve optimum results. Following a final wash step, the cells were resuspended in 1% paraformaldehyde.

Flow cytometry analysis

All data were acquired on a FC500 flow cytometer instrument within 8 hours of staining and was analyzed using CXP Cytometer software (Beckman Coulter Inc, Fullerton, CA). The cells were gated initially on lymphocytes as determined by forward and side scatter, and where possible, data from at least 100,000 small lymphocytes were collected. For most samples, 10,000 to 20,000 CD3+ CD8+ T cells were collected for analysis.

Quantitation of L-selectin^{low} CXCR3+ effector T cells

The number of total lymphocytes per liter (N) was analyzed on an XE2100 automatic blood cell analyzer. The frequency of L-selectin^{low} CXCR3+ proinflammatory effector T cells (F) was acquired by flow cytometry. The number of L-selectin^{low} CXCR3+ proinflammatory effector T cells per liter (T) was calculated as follows: $T = N \times F$.

Statistical analysis

The mean and standard deviation were used to describe the samples. Analyses were performed using SPSS version 13.0

(SPSS Inc, Chicago, IL). Significant differences between patients and healthy volunteers were assessed using one-way analysis of variance and least squares difference for post hoc multiple comparisons. Differences between pretreatment and post treatment for arterial and venous samples were analyzed using the paired-sample *t*-test. All mononuclear cell samples were blinded as to the source of cells. Information on study participants was compiled only after all the experiments were completed and the data were prepared for analysis.

Results

To analyze the influence of transcatheter arterial chemoembolization on expression of L-selectin and CXCR3 on T cells, we first compared the phenotype of CD8 T cells in patients with hepatocellular carcinoma prior to treatment with that of healthy controls. In patients with hepatocellular carcinoma, L-selectin-CXCR3+ effector T cells dominated in the peripheral vein, with a vast majority of $74.47\% \pm 9.36\%$ compared with L-selectin^{high} CXCR3- and L-selectin^{low} CXCR3+ T cells. In contrast, the frequency of L-selectin^{high} CXCR3- proliferative T cells was $52.78\% \pm 11.83\%$ in a healthy volunteer, significantly higher than that of L-selectin^{low} CXCR3+ T cells ($17.51\% \pm 5.91\%$), and L-selectin-CXCR3+ T cells ($17.02\% \pm 7.49\%$, $P < 0.05$, Figure 1).

Next, we tracked changes in L-selectin and CXCR3 expression on T cells after transcatheter arterial chemoembolization. The frequency of L-selectin^{low} CXCR3+ T cells

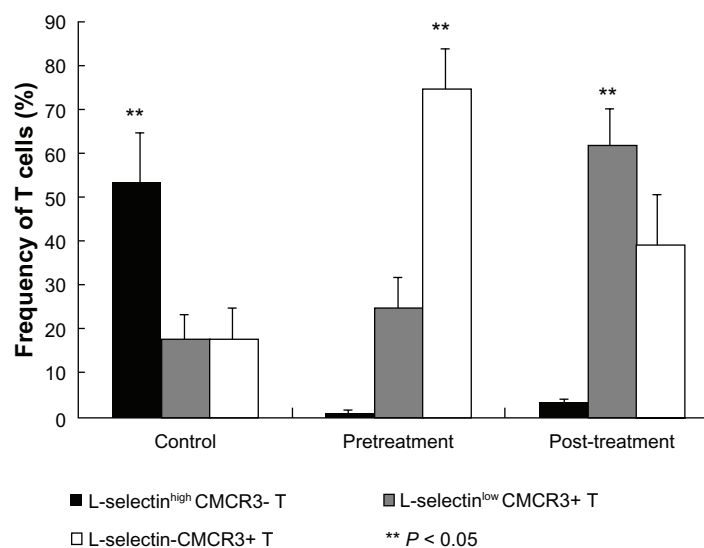


Figure 1 Comparison of expression of L-selectin and CXCR3 on CD8 T cells between patients with hepatocellular carcinoma and healthy volunteers.

Notes: In patients with hepatocellular carcinoma, the frequency was $0.6\% \pm 0.25\%$ for L-selectin^{high} CXCR3- T cells, $24.45\% \pm 7.36\%$ for L-selectin^{low} CXCR3+ T cells, and $74.47\% \pm 9.36\%$ for L-selectin-CXCR3+ T cells. The proportion of L-selectin-CXCR3+ T cells was significantly higher than that of the other two subsets (both $P < 0.05$). In contrast, the frequency of L-selectin^{high} CXCR3- T cells in healthy volunteers was $52.78\% \pm 11.83\%$, significantly ($P < 0.05$) higher than for L-selectin^{low} CXCR3+ T cells ($17.51\% \pm 5.91\%$) and L-selectin-CXCR3+ T cells ($17.02\% \pm 7.49\%$). In patients with hepatocellular carcinoma, the frequency of L-selectin^{low} CXCR3+ T cells increased to $61.92\% \pm 8.69\%$ posttreatment, which was significantly higher than the $24.45\% \pm 7.36\%$ value pretreatment ($P < 0.05$); the percentage of L-selectin^{high} CXCR3- T cells posttreatment remained unchanged, while L-selectin-CXCR3+ T cells decreased from $74.47\% \pm 9.36\%$ to $39.08\% \pm 11.46\%$. The mean value \pm standard deviation of percentage of T cells is shown.

increased to $61.92\% \pm 8.69\%$ after treatment, which was significantly higher than before treatment ($P < 0.05$, Figure 1), while L-selectin-CXCR3+ T cells decreased from $74.47\% \pm 9.36\%$ to $39.08\% \pm 15.46\%$. The proportion of L-selectin^{high} CXCR3- T cells remained unchanged. It is suspected that as one cell population increases, the other decreases, and vice versa. Because of this limitation, it is difficult to determine which event, ie, an increase in L-selectin^{low} CXCR3+ T cells or a decrease in L-selectin-CXCR3+ T cells, is the initial one. Therefore, we analyzed absolute numbers of T cells to confirm the above observation. Similar to the percentages, the absolute number of L-selectin^{low} CXCR3+ T cells after treatment increased to $18.98 \pm 2.33 \text{ e7/L}$, which is significantly more than the $6.10 \pm 1.21 \text{ e7/L}$ prior to treatment ($P < 0.001$). L-selectin-CXCR3+ T cells wavered between $13.79 \pm 3.78 \text{ e7/L}$ pretreatment and $13.68 \pm 2.47 \text{ e7/L}$ after treatment, and L-selectin^{high} CXCR3- T cells from $0.13 \pm 0.06 \text{ e7/L}$ to $0.87 \pm 0.25 \text{ e7/L}$, respectively, with no significant difference between pretreatment and post treatment values (Figure 2).

It was unclear whether the amounts of L-selectin^{low} CXCR3+ T cells increased because of redistribution caused by transcatheter arterial chemoembolization. To address this issue, we examined the expression of CXCR3 and L-selectin on T cells in the peripheral vein and in the hepatic artery. The frequency of L-selectin^{high} CXCR3- T cells in the hepatic artery and the peripheral vein was $11.31\% \pm 10.92\%$ and $12.55\% \pm 12.4\%$, respectively, the frequency of

L-selectin^{low} CXCR3+ T cells was $22.64\% \pm 12.63\%$ and $21.85\% \pm 13.61\%$, and the frequency of L-selectin-CXCR3+ T cells was $45.25\% \pm 12.45\%$ and $44.25\% \pm 13.77\%$, respectively (Figure 3). There was no significant difference in T cell phenotype between the artery and the vein.

It could be that transcatheter arterial chemoembolization induced proliferation and differentiation of T cells. Therefore, we next tested CD69 expression on T cells. The frequency of CD69+ T cells before treatment was $2.53\% \pm 0.51\%$ in the artery and $2.38\% \pm 0.49\%$ in the vein, respectively, and post treatment was $3.80\% \pm 0.62\%$ and $4.48\% \pm 0.75\%$, respectively (Figure 4). The proportion of activated T cells after treatment was significantly higher than that before in both the artery and the vein ($P < 0.05$). Interestingly, there was no significant difference between their frequency in the artery and vein before and after treatment.

Discussion

Transcatheter arterial chemoembolization improves survival in patients with hepatocellular carcinoma, for whom curative therapies are not available. However, so far, the underlying immune mechanism is still unclear. Our study affords a longitudinal observation of the alteration of subsets of memory T cells during transcatheter arterial chemoembolization. In healthy volunteers, L-selectin^{high} CXCR3- proliferative T cells were present in considerably higher proportions than was the effector subset of cells, suggesting a powerful potential to proliferate, expand, and maintain immune homeostasis

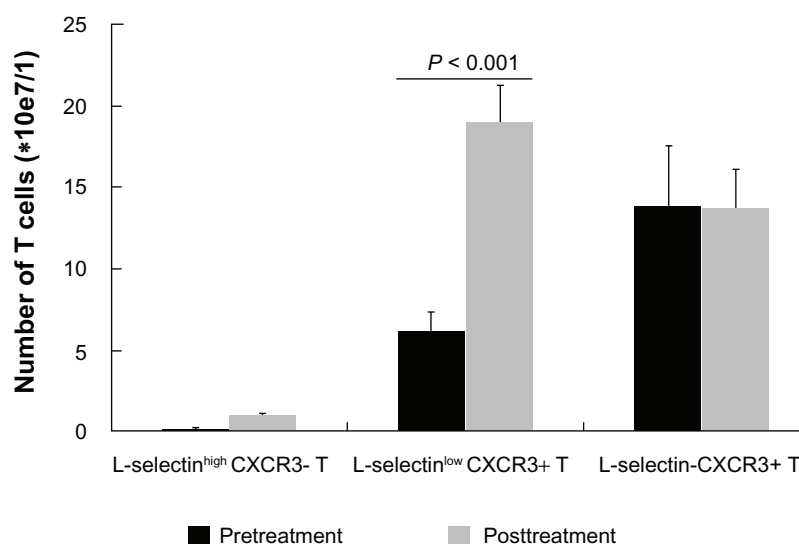


Figure 2 Change in the absolute number of L-selectin^{low} CXCR3+ T cells before and after transcatheter arterial chemoembolization.

Notes: In patients with hepatocellular carcinoma, the number of L-selectin^{low} CXCR3+ T cells posttreatment was $18.98 \pm 2.33 \text{ e7/L}$, which is significantly more than the $6.10 \pm 1.21 \text{ e7/L}$ prior to treatment ($P < 0.001$). L-selectin-CXCR3+ T cells changed slightly from $13.79 \pm 3.71 \text{ e7/L}$ pretreatment to $13.68 \pm 2.47 \text{ e7/L}$ after treatment, and L-selectin^{high} CXCR3- T cells from $0.13 \pm 0.06 \text{ e7/L}$ to $0.87 \pm 0.25 \text{ e7/L}$, respectively, with no significant difference between pretreatment and posttreatment. The mean value \pm standard deviation of number of T cells is shown.

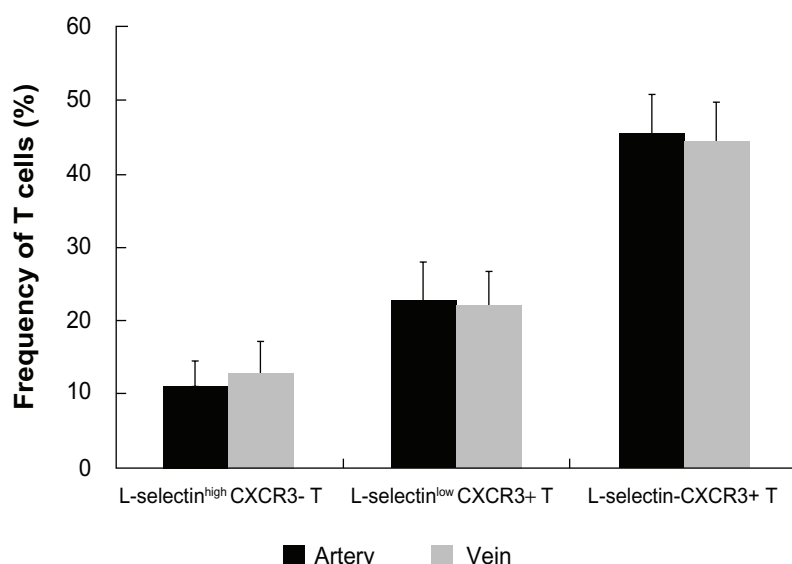


Figure 3 CXCR3 and L-selectin expression on T cells in the peripheral vein and the hepatic artery.

Notes: In patients with hepatocellular carcinoma, the frequency of L-selectin^{high} CXCR3- T cells was 11.31% ± 10.92% and 12.55% ± 12.4% in the artery and vein, and of L-selectin^{low} CXCR3+ T cells was 22.64% ± 12.63% and 21.85% ± 13.61% in the artery and vein, respectively. There was no significant difference in the phenotype of T cells between the artery and the vein. The mean value ± standard deviation of percentage of T cells is shown.

upon encountering an antigen challenge, given that naive T cells and central memory T cells in a steady-state home to high endothelial venules and T cell areas in secondary lymphoid tissue via expression of the L-selectin molecule.¹¹ In contrast, the frequency of L-selectin-CXCR3+ effector T cell subsets was higher in patients with hepatocellular carcinoma before treatment. However, both the absolute numbers and proportions of L-selectin^{low} CXCR3+ proin-

flammatory T cell subsets increased markedly post treatment compared with pretreatment, being consistent with our recent finding that patients with chronic hepatitis B and a viral response showed a significantly higher proportion of CXCR3+CD8+ T cells pretreatment and at 24 weeks.⁸ An increase in L-selectin^{low} CXCR3+ proinflammatory effector T cell subsets may be due to two factors, one of which may involve the T cell compartment. However, interestingly, there

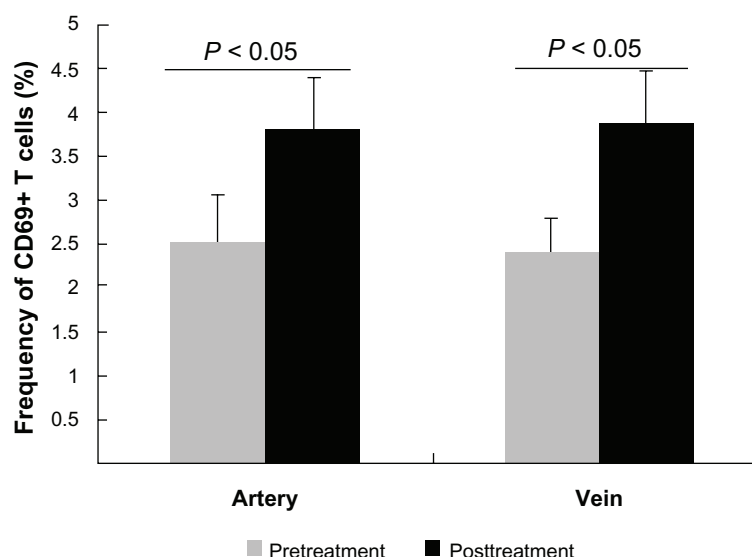


Figure 4 Comparison of CD69 expression on T cells before and after treatment.

Notes: The frequency of CD69+ T cells in patients with hepatocellular carcinoma before treatment was 2.53% ± 0.51% and 2.38% ± 0.49% in the artery and vein, and posttreatment was 3.80% ± 0.62% and 4.48% ± 0.75%, respectively. The proportion of activated T cells posttreatment was significantly higher than that before treatment, both in the artery and the vein ($P < 0.05$). There was no significant difference between its frequency in the artery and vein before or after treatment. The mean value ± standard deviation of percentage of T cells is shown.

was no significant difference in the frequency of T cell subsets between the peripheral vein and the hepatic artery, indicating that the higher proportion of the L-selectin^{low} CXCR3+ T cell subset posttreatment did not come from redistribution of T cells, which is in agreement with the finding of Thimme et al that the frequency of alpha-fetoprotein-specific CD8 T cells in the livers of patients with hepatocellular carcinoma was not higher than in peripheral blood.¹² The other factor may be T cell proliferation. Necrosis resulting from chemoembolization can lead to stimulation of immunity^{13–17} because necrotic cells release proinflammatory molecules, such as high mobility group box protein, uric acid, and heat shock protein 70, which can trigger dendritic cells to mature. Mature dendritic cells stimulate CD4+ T cells to provide help for CD8+ T cells, encouraging their expansion into effector cells. Moreover, inflammatory stimuli produced by necrotic tissue are able to induce high endothelial venule luminal CXCR3 ligands and L-selectin ligand expression quickly in the high endothelial venule lumen in reactive nodes,^{18,19} thus L-selectin^{low} CXCR3+ proinflammatory CD8+ T cells preferentially traffic to reactive nodes, where they undergo homeostatic proliferation to boost T numbers.²⁰

Furthermore, enhanced frequency of T cells expressing the CD69 molecule following treatment further demonstrated that amounts of activated T cells increased post transcatheter arterial chemoembolization. Once expressed, CD69 acts as a costimulatory molecule for T cell activation and proliferation.²¹ Increasing evidence has demonstrated that the phenotype of memory T cells can alter due to activation, homeostasis, and recirculation.^{8,22,23} L-selectin expression on memory T cell subsets seems to be interconvertible, with L-selectin^{high} cells losing L-selectin expression following antigen activation or in response to homeostatic cytokines.^{24–26} It is also known that stimulation with CXCL12 induces a spatial association between CXCR4 and the T cell receptor complex, allowing the chemokine receptor to activate components of the T cell receptor signaling pathway, which results in increased CD69 and interleukin-2 production,²⁷ and a similar utilization of the T cell receptor signaling pathway has been shown for CXCR3.²⁸ Stimulation of CXCR3 on the surface of activated T cells produces signals which can synergize with those transduced by activation of the T cell receptor complex. These signals enhance both T cell proliferation and specific migration of T cells across cytokine-activated endothelium.²⁹

Here we present the alteration of L-selectin and CXCR3 expression on CD8+ T cells in the process of transcatheter

arterial chemoembolization. The amounts of L-selectin^{low} CXCR3+ proinflammatory effector T cells increase significantly following treatment with transcatheter arterial chemoembolization. Because successful immunotherapy to a large extent seems to be reduced to the issue of creating an appropriate inflammatory reaction, an increase in proinflammatory L-selectin^{low} CXCR3+ T cells may be one of the factors critical to induction of tumor immunity.

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Disclosure

The authors report no conflicts of interest in this work.

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