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THE NORMAL AND METASTASES-BEARING LIVERS RETAIN VARIOUS SPECIFIC SUBSETS OF LIVE LYMPHOCYTES FROM PORTAL CIRCULATION

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ABSTRACT

The liver is among the organs that trap lymphocytes flowing through their blood vasculature. These cells, marginated in sinusoids, participate in the liver's anti-viral and anti-tumor processes. The molecular mechanism of this lymphocyte margination and cooperation with resident sinusoidal cells remains obscure and inadequately studied due to the difficulties in obtaining samples of sinusoidal blood from a living animal. To overcome these shortcomings, we have worked out an in situ *rat liver perfusion model in exsanguinated animals that enables quantitative observations of blood lymphocyte trapping in sinusoids. The cell populations trapped by the liver and retained in the perfusing blood were characterized with respect to their phenotypes and cytotoxicity. Perfused livers, previously washed out of sinusoidal lymphocytes, halted leukocytes from normal perfusing blood. The numbers of halted post-perfusion $CD5^+$, $CD4^+$, $CD8^+$, $CD56^+$ (ED1) and MHC class II^+ (OX6) subsets did not differ statistically from the pre-perfusion population, which suggests active extraction of leukocytes during perfusion. Moreover, cytotoxicity of post- and preperfusion populations against CC531 and K562 remained at a similar level. The perfused livers with CC531 colon adenocarcinoma metastases halted higher numbers of the CD14 and MHC class II^+ and fewer of $CD11b^+$ and $CD54^+$ normal blood leukocytes than normal livers. The phenotypes of cells retrieved from sinusoids after perfusion were almost identical to those obtained prior to perfusion. Interestingly, the post-perfusion populations displayed higher cytotoxic capacity than before perfusion. Taken together, the* in situ *liver perfusion method allows the study of the specificity and kinetics of recruitment of specific populations of host leukocytes in metastatic tumor tissue and evaluation of their cytotoxicity levels.*

Recent observations indicate that the liver, similarly to the lymphoid organs, is a site where specific immune cell-mediated responses by recruited lymphocytes can take place (1,2). In addition to the resident Kupffer and endothelial cells, there are specific subsets of marginating portal blood mononuclear cells that participate in the intrahepatic immune processes. Blood flushed from the liver venous vasculature contains populations transiently halted in the sinusoids. These cells adhere to Kupffer and sinusoidal cells on liver tissue cryosections (3). They belong to the NK (natural killer) population (4-7) and are cytotoxic toward tumor cells *in vitro* and *in vivo* (8,9). There are also subsets of T-lymphocytes (10,11) which participate in the host-versus-graft (12,13) and graft-versus-host reaction (14).

The role of sinusoidal T lymphocytes (15,16) in allogeneic transplantation tolerance remains unclear. Specific studies needs to be undertaken to determine the definitive role of the liver sinusoidal blood lymphocytes, in normal conditions and disease. The *in vivo* investigations, based on intravenous administration of labeled blood lymphocyte subsets and following their migration to the liver, are burdened by trapping of the infused cells in the spleen and lymph nodes, prior to their "homing" to the liver (7). Moreover, the quantitative evaluation of "homing" and topographical location of trapped leukocytes cannot be analyzed without sequential washing-out of liver sinusoidal blood and cryosectioning of liver tissue. *In vivo* harvesting of portal blood leukocytes requires cumbersome cannulation of portal and hepatic veins and manipulation of the liver, causing major disturbances in splanchnic blood flow and liver blood supply, totally distorting sinusoidal blood perfusion and lymphocyte margination. These difficulties can be overcome by the *in situ* perfusion of the isolated liver using a perfusion circuit, maintaining basic hepatic biochemical, scavenging and blood lymphocyte trapping functions. We have developed a rat liver perfusion model for studying the mechanism of specific extraction of blood lymphocytes (17). In this model of short ischemia time, minimal liver touch, no dissection *in situ* blood-perfused liver, the populations flushed from the normal liver vasculature after perfusion remained phenotypically and functionally identical to those retrieved from livers prior to perfusion.

In this study the following questions were asked: 1) which blood cytotoxic cell populations are sequestered during perfusion in sinusoids of normal livers and livers with CC531 adenocarcinoma metastases, and 2) which adhesion molecules and their ligands play a role in blood cell trapping in normal livers and in the metastatic foci?

MATERIALS AND METHODS

Animals

Wistar (W/Wag) ($RT1^a$) rats weighing 200-300 g, from our animal husbandry were used. Animals were treated humanely using approved procedures in accordance with the guidelines of the Institutional Animal Use and Care Committee.

Liver Perfusion Circuit

The liver perfusion circuit consisted of a peristaltic pump (Unipan, type 371, Poland), oxygenator with $0.1m^2$ gas exchange surface (Merasilox, Senko Medical, Japan) and heat-exchanger. A water thermostat (LKB, type 2209 Multicool, Sweden) maintained the perfusate and liver temperature at 37°C.

Liver Perfusion Technique

Rats were anesthetized with ether, heparinized i.v. with 400 I.U. of heparin (Novo Nordisk, Norway) and exsanguinated. A no-dissection, no-liver-touch, *in situ* liver perfusion technique was applied. Cannulation of the portal vein and perfusion and of the suprahepatic vena cava for collection of liver perfusate was carried out. The infrahepatic vena cava was then ligated as were all small veins in the ligaments fixing the liver to the diaphragm and retroperitoneum. As the first step, livers were washed out through the portal cannula with 40 ml of Hanks' solution (Gibco, USA) at 22°C and under 15 cm H_2O pressure. The washout fluid was collected for further studies. The washout cells were termed liver-associated-lymphocytes (LAL). Then the liver was connected to the circuit and perfused for 60 minutes under 12-15 cm H_2O pressure. The warm ischemia time, from the moment of sacrificing the rat until starting perfusion, lasted 10-15 minutes. Following completion of perfusion, the liver vasculature was again washed out, using the same technique as before perfusion, and the washout fluid was collected.

Flow-Cytometric Phenotypic Assessment of Cells Isolated From Liver Sinusoids

For identification of cellular antigens CD4, CD5, CD8, CD14, CD56, MHC class II, CD54, CD11a, CD11b and CD18, a direct immunofluorescence staining method was applied using specific mouse monoclonal antibodies (Serotek, UK) and immunoglobulins for isotypic controls. Cells (10^6) in 100µl PBS + 2% FCS were incubated for 30 min at +4°C in the presence of 10µl of monoclonal antibody conjugated with phycoerythrine: W3/25PE (CD4) – helper/inducer cells, OX8PE (CD8) – suppressor/cytotoxic cells, OX19PE (CD5) – T cells, 3.2.3PE (CD56) – NK cells, ED1PE (CD14) – monocytes, OX6PE – MHC class II positive cells, 4C9PE (CD54) – ICAM-1, WT-1PE (CD11a) – LFA-1α, OX-42PE (CD11b) – Mac-1 α, WT-3PE (CD18) – Mac-1ß or LFA-1 ß. After washing, cells were suspended in 0.5ml 1% paraformaldehide and analyzed in flow cytometer FACStar (Becton & Dickinson, USA). Statistical analysis was performed using FlowMATE computer software (Dako, Denmark)

Cytotoxicity of Washed-out LALs and Perfusate Cells

The CC351 (1,2- dimethylhydrazine-induced colon adenocarcinoma, syngeneic with WAG/Rij rats) and K652 (human erythromyeloid leukemia) cells at a concentration of $1x10^6$ were labeled with 1.0µCi ^{51}Cr (Amersham, UK), mixed with effector cells at E/T ratios 40:1, 20:1, 10:1, and incubated for 18h. The percentage of cytotoxicity was calculated.

Tumor Induction

Liver tumors were induced by injection of $1x10^6$ of CC531 colon cancer cells into the portal vein. CC531 is a 1,2-dimethylhydrazine-induced, moderately differentiated and weakly immunogenic adenocarcinoma of the colon, syngeneic with WAG/Rij rats (12). The cells were kindly provided by Dr. P. J. K. Kuppen, Leiden University Medical Center, Leiden, and Dr. R. L. Marquet, University Hospital-Dijkzigt, Rotterdam, The Netherlands. The total liver tumor mass was on the average 15.1g after 3 weeks (range from 5.2 to 20.7g; mean 56.5% of total liver weight).

Experimental Groups

The experiments were carried out in 2 groups. In group 1 (n=9), normal livers were perfused with normal blood (controls) and in group 2 (n=9) tumor-bearing livers were perfused with normal blood. The pre-perfusion washout population was termed LAL1, the post-perfusion population LAL2. The population from the normal liver was marked with the letter n, from tumor-bearing liver with the letter t.

Statistical Evaluation

The analysis was carried out in two steps. In the first step, mid-values of parameters of interest were compared using One-Way Anova or, in the case of non-normality of the distribution, Kruskal-Wallis test. In the second step, if statistically significant differences were observed, the following multiple comparison tests were used: 1) Dunnet's or (in case of non- normal distribution) Dunn's, if the differences with the selected group were the objective of the study; 2) Tukey's or (in case of non normal distribution) Dunn's for all pairwise comparisons.

RESULTS

LAL Counts in Liver Sinusoidal Washout Before and After Perfusion

The mean cell count before normal liver perfusion was $1.04\pm0.2x10^6$ per g, and after 60 min of perfusion it was $0.9\pm0.1x10^6$ per g of liver tissue. The cell count retrieved from

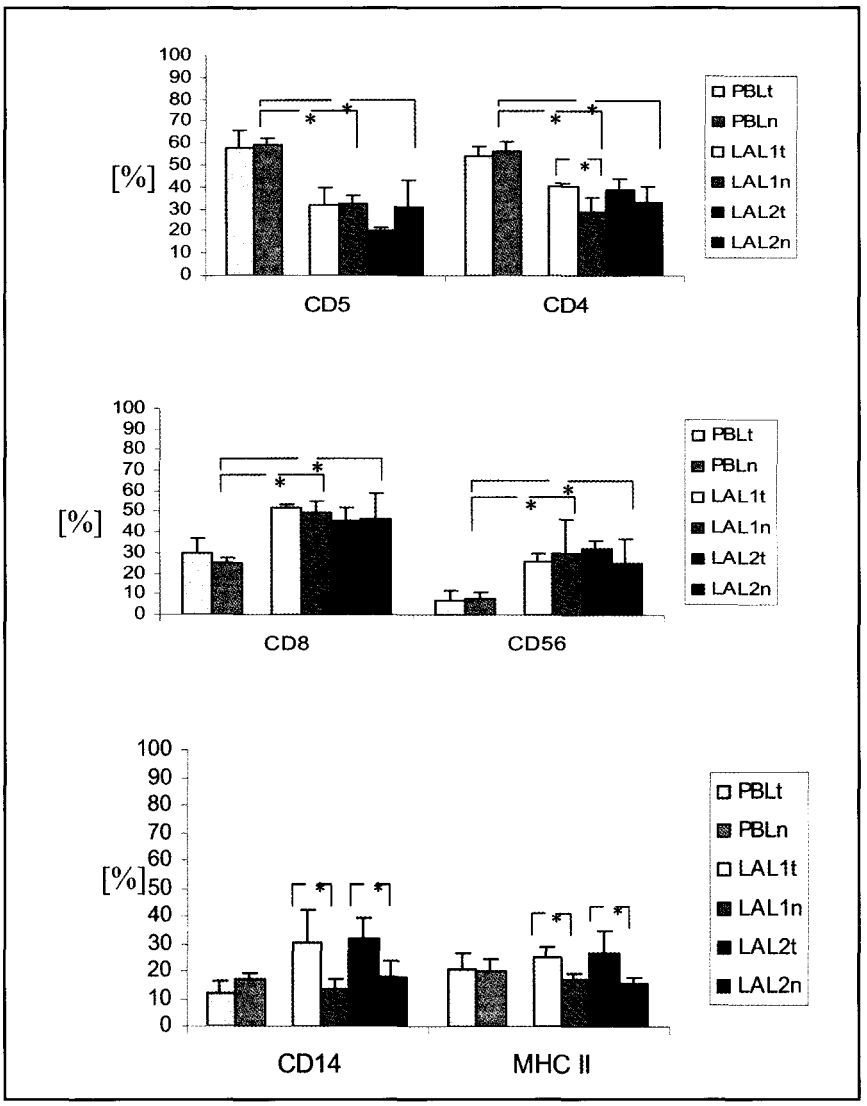

*Fig.1. Phenotypes of peripheral blood mononuclear (PBL) and liver sinusoidal washout cells from normal (n) and tumor-bearing (t) rats. LAL 1n, t – liver associated leukocytes washed out from normal (n) and tumoral (t) livers before perfusion, LAL 2n, t – leukocytes washed out after 60 min perfusion with normal blood. Values are mean percentage ± SD, * $p < 0.05$. Middle).*

tumorous livers did not differ from that of normal livers.

Group 1. Normal Livers

Phenotypes of normal liver sinusoidal washout LAL and PBM in normal rats

The sinusoidal washout LAL1n population obtained prior to perfusion contained 32.5±4.0% of $CD5^+$ (T cells), 29.1±6.1% of $CD4^+$, 49.7±5.1%of $CD8^+$, 30.1±16.3% of $CD56^+$, 13.6±3.6% of CD14, and 16.7±2.5% of MHC class II^+ cells (*Figs. 1-3*). The LAL1n population differed from the perfusing blood PBMn (PBLn). There were fewer $CD5^+$ and $CD4^+$ cells in LAL1n, and more $CD8^+$ and $CD56^+$ cells

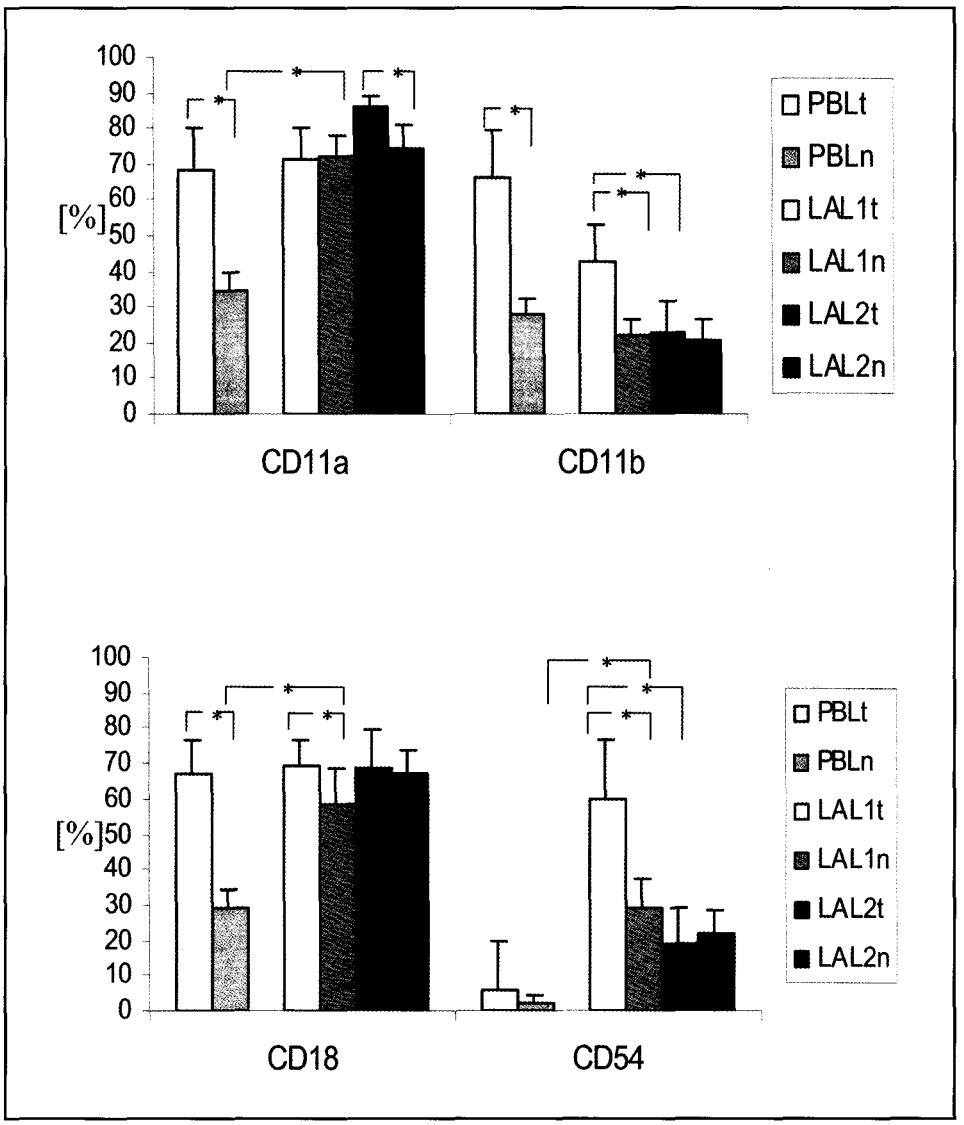

Fig. 2. Adhesion molecules on peripheral blood mononuclear and liver sinusoidal washout cells. For explanation of abbreviations, see Fig. 1.

($p<0.05$), which is physiological. *(Fig. 1 upper and middle)*. After 60 min perfusion, the retrieved washout population (LAL2n) was quantitatively the same as LAL1n *(Fig. 1)*. This indicates that the perfused liver specifically sequestered the same populations as did a normal living rat liver.

Adhesion molecules on normal liver sinusoidal washout LAL and PBM

In the LAL1n population, 72.0±5.8% of $CD11a^+$, 21.7±4.6% of $CD11b^+$, 58.5±9.8% of $CD18^+$ and 29.0±7.9% of $CD54^+$ cells were found *(Fig. 2)*. The LAL1n values were higher than in PBMn (PBLn) population for the $CD11a^+$, $CD18^+$ and $CD54^+$ cells ($p<0.05$). In the post-perfusion LAL2n population the values were statistically not different from LAL1n.

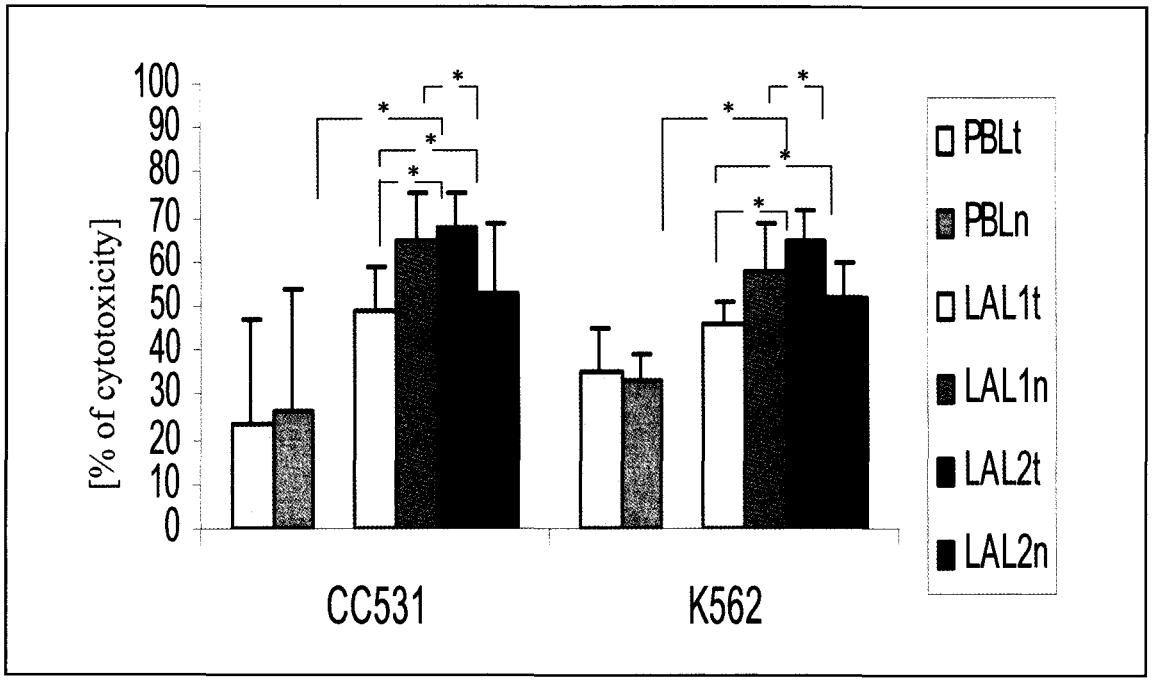

*Fig. 3. Cytotoxic activity of blood mononuclear and sinusoidal washout cells against CC531 and K562 cells. Mean percent of cytotoxicity ± SD, * p<0.05. For explanation of abbreviations, see Fig. 1.*

Cytotoxicity of sinusoidal washout LAL and PBM of normal rats to tumor cells

The level of cytotoxicity of the LAL1n to CC531 and K562 cells was at all E/T ratios significantly higher than of PBMn (PBLn) ($p<0.05$) *(Fig. 3)*. No difference in the cytotoxicity level between the LAL1n and LAL2n was found after perfusion, indicating active halting of cytotoxic cells in the sinusoids of perfused livers.

Group 2.

Phenotypes of sinusoidal washout LAL and PBM in liver CC531 tumor-bearing rats

The LAL1t population contained significantly more CD4+, CD14+ and MHC class II+ cells than the LAL1n population ($p<0.05$) *(Fig. 1)*. The PBLn and PBLt populations did not significantly differ in the distribution of leukocyte phenotypes. The post-perfusion LAL2t population did not differ from LAL1t population, which suggested active retention of specific populations of perfusing blood cells also by the tumorous liver.

Adhesion molecules on sinusoidal washout LAL and PBM in liver CC531 tumor-bearing rats

The LAL1t contained significantly more CD11b+, CD18+ and CD54+ cells than LAL1n ($p<0.05$) *(Fig. 2)*. More CD11a, 11b and 18 cells were found in PBMt than PBMn ($p<0.05$). After perfusion, there was significantly fewer CD11b+ and CD54+ cells in the LAL2t than LAL1t population ($p<0.05$) *(Fig. 2)*.

Cytotoxicity of sinusoidal washout LAL and PBL in liver CC531-bearing rats

The cytotoxicity level of LAL1t was significantly lower than of LAL1n ($p<0.05$), whereas that of LAL2t was evidently higher than of LAL1t ($p<0.05$) *(Fig. 3)*. The latter could be interpreted in terms of active extraction of cytotoxic cells by the perfused liver.

DISCUSSION

The liver normally recruits specific populations of blood mononuclear cells and retains them in the sinusoids (4,18,19). Approximately one million cells per gram of liver tissue, attached to the sinusoidal lining, can be washed out from the sinusoids. It was found that leukocytes retrieved from the rat sinusoids contain more $CD8^+$ and $CD56^+$ and fewer $CD5^+$ and $CD4^+$ cells than peripheral or portal blood (4,20–23). The CD4/CD8 ratio for the liver sinusoidal leukocytes is 1:3.5 compared to 2:1 in blood (23,24). It has been documented that rat liver sinusoidal leukocytes labeled with the 3.2.3 antibody reveal strong cytotoxic activity against tumor cells, both *in vitro* and *in vivo* (21,25–28).

In this study, after 60 min of perfusion of normal livers with blood, the previously emptied vasculature became repopulated with leukocytes from the perfusate. The post-perfusion washout revealed the presence of cells (LAL2n) of the same phenotypes and distribution as the pre-perfusion washout (LAL1n) but different from the perfusing blood (PBLn).

The *ex vivo* perfused livers with CC531 metastases sequestered higher numbers of the CD14 (ED1) and MHC class II^+ (OX6) and fewer $CD11b^+$ and $CD54^+$ normal blood leukocytes than normal livers. Blood cells sequestered in the perfused tumoral liver displayed higher cytotoxic capacity than before perfusion. The high numbers of cells of $CD14^+$ and MHC class II^+, a phenotype with high cytotoxic activity, which were trapped in the liver *in vivo* and during perfusion should be ascribed to the liver tumor intrinsic factors and not to the blood phenotypic pattern as blood of tumor-bearing rats had a leukocyte phenotypic pattern almost identical to that of normal rats (PBLt=PBLn).

Elucidation of the mechanism of preferential trapping of certain subsets of blood leukocytes in the liver with metastatic tumor led to studies of adhesion molecules and their ligands. We have previously studied expression of these molecules on tumor cells. The CC531 cells did not express any adhesion molecules, and the endothelial cells of tumor tissue were difficult to identify due to irregularities in the vessel shape and distribution, although some of them stained strongly with the anti-HIS 52 and anti-CD31 antibodies. The CD54 molecule was expressed only occasionally (data not presented). In the present study, the perfused tumor bearing livers sequestered as many CD11a and CD18 cells as a normal liver but fewer CD11b and CD54 cells. These data suggest that the molecules studied did not play a major role in lymphocyte trapping.

In our experimental model, two mechanisms of leukocyte recruitment in the perfused liver may be operating. One is the "physiological" sequestering of $CD8^+$ and $CD56^+$ cytotoxic subset as described for normal liver (22). These populations have a predilection to "home" to the liver when administrated intravenously (29,30). The other mechanism is specific attraction of leukocytes mediated by the proliferating tumor cells but this process is unclear.

Taken together, the *in situ* liver perfusion method allowed the documentation of the specificity and kinetics of recruitment of specific populations of host leukocytes in the tumor tissue and evaluation of their cytotoxicity levels but left open the question of adhesion molecules responsible for lymphocyte trapping. Nevertheless, this model is suitable for further studies on the mechanism of interaction of tumor and host infiltrating cells and how it is regulated.

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