Graft-versus-Leukemia Reactivity Involves Cluster Formation between Superantigen-reactive Donor T Lymphocytes and Host Macrophages

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ABSTRACT

T-cell-mediated antitumor effects play an important role clinically in allogeneic graft-versus-leukemia (GvL) reactivity, whereas T-cell-mediated antihost effects are associated with a risk of developing graft-versus-host (GvH) disease. GvL and GvH were compared in an animal tumor model system after the systemic transfer of allogeneic antitumor immune T lymphocytes from B10.D2 [H-2b] mice into ESb-MP tumor-bearing or normal DBA/2 (H-2b; Mls\(^b\)) mice. Here we demonstrate that this T-cell-mediated therapy involves the formation of clusters of donor CD4 and CD8 T cells with host macrophages, in particular, with a subpopulation expressing the lymphocyte adhesion molecule sialoadhesin. DBA/2 mice and the derived tumor ESb-MP express viral superantigen 7 (Mls\(^b\)), an endogenous viral superantigen that is absent from B10.D2 mice. To test the contribution of viral superantigen 7-reactive VB6 donor T cells in the GvL-mediated eradication of liver metastases, we performed immunohistological and transmission electron microscopy studies. VB6+ CD4 and CD8 T cells from B10.D2 donors formed tight clusters with host sialoadhesin-positive macrophages, and transmission electron microscopy pictures revealed direct membrane-membrane interactions between T cells and macrophages. Clusters were more abundant and consisted of more cells in tumor-bearing hosts (GvL model) than in non-tumor-bearing hosts (GvH model). In addition, VB6 T cells within the clusters showed a strong proliferation activity, indicating stimulation. Moreover, in an in vitro tumor cytostasis assay, primed as well as nonprimed purified VB6 T cells from donor mice were able to inhibit the proliferation of superantigen-expressing ESb-MP lymphoma cells. This suggests that the transferred superantigen-reactive VB6 T cells contribute to the eradication of metastases. The observed cell clusters might be sites for antigen presentation and the activation of tumor-reactive T cells.

INTRODUCTION

The transfer of activated tumor-reactive lymphocytes into tumor-bearing animals has been reported as an effective ADI against clinically established metastases (1-3). T-cell-mediated antitumor effects also play an important role in the so-called GvL effect that is usually associated with a risk for the development of GvH disease with significant morbidity and mortality (4). We have established an animal model for the investigation of the GvL and GvH reactivity of immune lymphocytes. The murine leukemia system consists of various well-defined metastasizing sublines (ESb, ESb-MP, and ESbLlacZ) that are derived from the chemically induced DBA/2 lymphoma L5178Y. The ADI effects are achieved by the transfer of in situ-activated tumor-reactive lymphocytes from the tumor-resistant strain B10.D2 into susceptible DBA/2 syngeneic mice. DBA/2 mice differ from B10.D2 mice not only in minor histocompatibility, but also in Mls antigens (5). These Mls determinants represent vSAGs encoded by MMTV proviruses that are integrated in the murine genome (6, 7). The possibility thus exists that the extraordinary resistance of B10.D2 (Mls\(^b\)) mice to DBA/2 (Mls\(^a\)) tumors may be due in part to a superantigen difference. This possibility is supported by our recent findings of a link between the absence of the endogenous MMTV superantigen vSAG7 and tumor resistance (5). vSAG7 behaves as a self-tolerogen in DBA/2 mice by deleting superantigen-reactive T cells with certain V\(\beta\) chains (e.g., VB6) from their repertoire (8). We have recently found that ESb tumor cells express not only individually distinct class I MHC (Kd)-restricted CTL epitopes (5, 9) but also express proviral Mtv-7 at the RNA level. One hypothesis to explain the mechanism of ADI is that upon ESb tumor cell transfer into B10.D2 mice, VB6+ T cells may become activated by the tumor-associated vSAG and, after

The abbreviations used are: ADI, adoptive cellular immunotherapy; GvL, graft-versus-leukemia; GvH, graft-versus-host; Mls, minor lymphocyte-stimulating antigen; vSAG, viral superantigen; MMTV, mouse mammary tumor virus; Sn, sialoadhesin; TAA, tumor-associated antigen; PO, horseradish peroxidase; AP, alkaline phosphatase; DC, dendritic cells; Th, T helper; APC, antigen-presenting cell; NO, nitric oxide; mAb, monoclonal antibody; IL, interleukin.

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4 Unpublished observations.
injection into tumor-bearing DBA/2 mice, may infiltrate ESb liver metastasis. Other results from this model show that a subset of host macrophages bearing the lymphocyte adhesion molecule Sn is able to process exogenous TAA from metastases and present it not only via MHC class II to CD4 cells (10) but also via class I to CD8 immune donor T lymphocytes. These macrophages could also present tumor-derived vSAG via their MHC II molecules, because in vivo studies have provided strong arguments for the paracrine transfer of vSAGs from cells that do not express MHC class II to MHC class I-positive cells proficient in vSAG presentation (11).

Therefore, the aim of this work was to study the possible physical interactions in situ between Sn+ host macrophages and V66 superantigen-reactive donor T cells in the liver of tumor-bearing mice during ADI by immunohistology. We demonstrate that upon transfer into tumor-bearing DBA/2 recipients, V66+ CD4 and CD8 T cells from B10.D2 donors form clusters a few days later with host Sn+ macrophages in metastasized livers. Tight membrane interactions between T cells and macrophages in these clusters will be shown by electron microscopy. Moreover, we will show that purified nonprimed V66 donor T cells are cytostatic in vitro for ESb tumor cells, suggesting that there are also in vivo antitumor effects after specific interactions of V66 T cells with a tumor-exposed superantigen.

MATERIALS AND METHODS

Animals

DBA/2 mice were obtained from IFFA Credo (Lyon, France), and B10.D2 mice were obtained from Olac (Bicester, United Kingdom). The animals were used at the age of 6–12 weeks.

Tumor Cell Lines

ESb cells represent a spontaneous highly metastatic variant of the chemically induced T lymphoma L5178Y (Eb) of DBA/2 mice. ESb-MP is an adhesion variant of ESb that grows in vitro attached to plastic, whereas ESb grows in suspension. In vivo, ESb-MP cells grow progressively but show a less aggressive phenotype, metastasizing more slowly than ESb cells and involving multiple organs (12).

Priming of B10.D2 Donor Mice and ADI of Recipient DBA/2 Mice (GVL and GvH)

DBA/2 mice were anesthetized with Rompun (0.1%; Parke, Davis & Co., Berlin, Germany). Ketanest (0.25%; Bayer, Leverkusen, Germany), and PBS at a volume of 1:1:3 to inject 2 × 10^7 ESb-MP tumor cells into the dermis of the shaved flank. To generate allogeneic immune effector cells, ESb-MP cells were injected i.v. at a dose of 10^7 cells into the tail vein of B10.D2 mice. Seven days later, the spleen cells were isolated and transferred at a dose of 2 × 10^7 cells/200 μl PBS into sublethally irradiated DBA/2 mice (5 Gy; 60Co source Gamma-Stratron F 80 S; Siemens, Braunschweig, Germany) that carried tumors (>1 cm in diameter) 3 weeks after intradermal tumor cell inoculation. Sublethally irradiated tumor-bearing DBA/2 mice in the control group remained untreated. To analyze GvH, non-tumor-bearing DBA/2 mice were irradiated and inoculated with the immune spleen cells.

Antibodies and Other Reagents

The following mAbs were used as culture supernatants: (a) rat-antimouse CD8 (clone Lyt-2; Ref. 13); (b) rat-antimouse V66 (clone 44-22-1; Ref. 14); (c) hamster-antimouse B7.1 [clone 16-10A]; Ref. 15; and (d) rat-antimouse Sn (clone SER4; Ref. 16). The first antibodies were visualized by using polyclonal donkey antirat and antihamster IgG (H + L) antibodies linked to PO and to AP (Dianova, Hamburg, Germany). A biotinylated rat antimouse antibody was used (Life Technologies, Inc., Eggenstein, Germany) for the immunohistochemical detection of CD4 T lymphocytes, and ExtrAvidin was linked to AP (Sigma, Deisenhofen, Germany).

Immunohistochemistry

Tissue Preparation. Livers were removed and snap-frozen in liquid nitrogen. Six-μm-thick consecutive cryostat sections were mounted on uncovered glass slides, air-dried overnight at room temperature, and fixed in acetone for 10 min at room temperature or air-dried for 1 h.

Single Staining. After fixation and drying, the slides were washed in PBS three times for 5 min. To avoid nonspecific binding, the sections were treated for 20 min with 1% normal mouse or rat serum, followed by an incubation with the first antibody for 45 min. After washing three times with PBS, the sections were incubated for another 45 min with the second antibody and washed before performing the substrate reaction for PO or AP. When using a biotinylated antibody, the slides were incubated with ExtrAvidin for 30 min. After staining, the sections were washed with water, counterstained with hemalaun (Mercer, Darmstadt, Germany), and mounted with glycerol gelatin (Merck). The same protocol was performed for negative controls in which either the first or the second antibody was omitted. All steps were performed in a humid chamber and at room temperature.

Double and Triple Staining. The staining procedure represents a combination of consecutive stainings for each antigen. Every single staining was completely finished with the substrate reaction before starting the second and third staining procedure, respectively. No counterstaining with hemalaun was performed.

Development of Enzyme Reactions. PO activity was revealed by incubating the sections in a solution containing 6 mg of 3-amino-9-ethylcarbazole (Merck) dissolved in 1.5 μl of N,N-dimethylformamide (Merck), 15 μl of 30% hydrogen peroxide, and 28.5 ml of 0.1 m acetate buffer (pH 5.0). The substrate for the development of AP consisted of 6.3 μl of 5% Neufucsin (Sigma) or 2 mg of Fast Blue (Sigma) in 16 μl of 4% sodium nitrite (Fluka, Buchs, Switzerland), 2 mg of naphthol AS-BI phosphate (Sigma) in 20 μl of N,N-dimethyformamide, and 3 ml of 0.05 mol/liter Tris-HCl buffer (pH 8.7) containing 1% H2O2.

Note

1 S. Muerkötter, M. Rocha, P. R. Crocker, V. Schirrmacher, and V. Umansky. Sialoadhesin-positive host macrophages play an essential role in graft versus leukemia (GVL) reactivity in mice: processing and presentation of exogenous (tumor) antigen via MMC class I, submitted for publication.
1 mM Levamisole (Sigma). The freshly prepared solutions were filtered and added to the sections. Development lasted about 3–5 min, with regular checking of the reaction intensity by microscopy. Immunohistochemical results were evaluated by counting the number of positively stained cells per liver lobule from three to six lobules/mouse. The means and SDs of the data obtained from two to three mice/time point were calculated and presented in the figures.

**Electron Microscopy**

On day 12 after ADI, DBA/2 mice were sacrificed, and liver samples of each (1 mm³) were cut and fixed immediately in 2.5% glutaraldehyde buffered with 0.05 M cacodylate and supplemented with ions (50 mM KCl and 2.5 mM MgCl₂) for 30 min at 4°C. After washing several times with 0.17 M cacodylate buffer, the samples were postfixed with 2% osmium tetroxide in 0.11 M cacodylate buffer for 2 h at 4°C. After washing several times with distilled water, samples were dehydrated with graded series of ethanol and propylene oxide and embedded in a mixture of Araldite and Epon resin. Ultrathin sections were cut and double-stained with a 1.5% ethanolic solution of uranylacetate for 20 min and with lead citrate as described by Reynolds (17) for 3 min.

**Flow Cytometric Analysis**

Spleen cells (1 × 10⁶) were washed in PBS supplemented with 5% FCS and treated with mAbs against mouse Fc receptors to avoid nonspecific binding, and then the staining for surface molecules was performed, which included the following antibodies: (a) FITC-labeled anti-Vβ6; (b) phycoerythrin-labeled anti-CD4; and (c) Red 613-labeled anti-CD8 (PharMingen, Hamburg, Germany). Intracellular staining for cytokines was performed as described previously (18). Briefly, after fixation in 4% formaldehyde (Merck), the cells were washed in PBS/FCS buffer containing 0.5% saponin (Sigma) and stained with one of the following FITC-conjugated mAbs: (a) anti-IL-2; (b) anti-IL-4; or (c) anti-IFN-γ (PharMingen). After washing once in 0.5% saponin-containing buffer and once in PBS/FCS, the cells were analyzed by flow cytometry using a FACScan with CELLQuest software (Becton Dickinson, Heidelberg, Germany). Typically, 30,000 events were collected, and the data were expressed as dot plots.

**Isolation of Vβ6+ T Cells**

B10.D2 mice were primed with 5 × 10⁴ ESB-MP cells injected intra-ear. After 7 days, single-cell suspensions from the spleens of primed and nonprimed mice were prepared by mechanical dissociation. After the lysis of erythrocytes by a short NH₄Cl hypotonic solution treatment, residual cells were washed twice and resuspended in PBS with 2% FCS. Cells were incubated with an anti-Vβ6 mAb (100 μM supernatant/10⁶ expected Vβ6+ cells) for 15 min on ice. After two washing steps, the cells were labeled with 60 μl/10⁷ cells of magnetic bead-conjugated antirat IgG isotype antibodies (MACS; Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) in 250 μl of PBS and 2% FCS for 15 min on ice. After washing, cells were collected in the column buffer (PBS containing 5 mM EDTA and 0.5% FCS). The depletion of labeled cells was achieved by passing the cells through a steel wool-containing MACS column that was attached to the MACS magnet (19). Vβ6− cells were washed out by an excess of column buffer, whereas the labeled Vβ6+ cells remained in the column. To obtain the positive selected cells, the column was removed from the magnet, and the cells were washed out with column buffer. To prove the purity of the positively selected cells, they were incubated with a FITC-conjugated anti-Vβ6 mAb (Life Technologies, Inc.) and measured by flow cytometric analysis. Cells were also stained with 1.5 μmol/liter propidium iodide to exclude dead cells. Flow cytometric analysis was performed using a FACScan (Becton Dickinson). Excitation laser frequency was 488 nm, and fluorescence emission was detected at 575 nm. Typically, 10,000 events were collected and analyzed.

**Cytostasis Assay**

ESB-MP tumor target cells were resuspended in medium (RPMI 1640; Life Technologies, Inc.) with antibiotics, 10% heat-inactivated FCS, 2 mM glutamin, 10 mM HEPES, and 50 μM 2-mercaptoethanol, and 5 × 10⁵ cells/20 μl medium/well were seeded in sterile 96-well round-bottomed plates (Renner, Dammstadt, Germany). To promote central adherence, the cells were incubated at 37°C for 2 h. Effector cells (Vβ6+ and Vβ6− spleen cells from both primed and nonprimed mice) were added in a volume of 180 μl with a final E:T ratio of 10:1. After 48 h of incubation at 37°C, 1 μCi of [³H]thymidine (Amersharm, Braunschweig, Germany) was added per well. Incorporated radioactivity was measured after 16–20 h in a liquid scintillation beta counter (LKB Wallac, Freiburg, Germany). Values from the experimental and control wells were compared to calculate the percentage of proliferation and the growth inhibition of tumor cells.

**RESULTS**

**Induction of Cytokine Production in the Spleen Cells of B10.D2 Mice upon Immunization with Tumor Cells.** First we studied the production of cytokines in the GvL effector cells of our model system by flow cytometry. Seven days after ESB-MP tumor cell injection, the spleens of B10.D2 mice were stained intracellularly with antibodies against IL-2, IL-4, and IFN-γ. Fluorescence-activated cell-sorting analysis of total lymphocytes revealed that the number of IL-2−, IL-4−, and IFN-γ-producing cells was 2-3-fold higher in primed mice than in nonprimed (control) mice (Table 1). To evaluate the contribution of the specific superantigen-reactive Vβ6+ T cells to cytokine production, double staining was performed with a mAb against the Vβ6 surface molecule, and intracellular staining was performed with anti-cytokine mAbs. As shown in Table 1, there was a large increase in Vβ6 IL-2-producing (10-fold) and IFN-γ-producing (8-fold) cells upon immunization, thus pointing toward a Th1-type cytokine response profile. Vβ6 T cells represented about 10% of the donor T-cell population.

**Eradication of Lymphoma Liver Metastasis after ADI as Visualized by the Disappearance of Ki67+stained Metastatic Foci.** B10.D2 anti-ESB-MP immune spleen cells, as described above, were transferred into ESB-MP tumor-bearing DBA/2 mice irradiated with 5 Gy. To evaluate the efficiency of such an ADI, livers from treated animals were removed 2 and 20 days after immune T-cell transfer (early and late stage) and
Table 1  Number of cytokine-producing cells

<table>
<thead>
<tr>
<th>Group</th>
<th>Total lymphocytes</th>
<th>Vβ6+ cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IL-2</td>
<td>IL-4</td>
</tr>
<tr>
<td>Nonprimed</td>
<td>1.4 ± 0.2</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>Primed</td>
<td>4.0 ± 0.6</td>
<td>0.2 ± 0.1</td>
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snap-frozen, and cryostat sections were stained with the proliferation marker Ki67, which recognizes a nuclear antigen present in proliferating cells (20). As shown in Fig. 1A, 2 days after the transfer, livers from tumor-bearing mice contained groups of proliferating tumor cells, which were spreading from the portal vein into the parenchyma. At 20 days after therapy (Fig. 1B), only some proliferating cells remained in the liver. These were not in clusters, as were the metastatic tumor foci, and could represent proliferating donor T cells. In addition to other parameters that have been reported previously (4), the disappearance of tumor foci demonstrates the efficiency of this therapy.

CD4 and CD8 Donor T Cells Form Clusters with Host Sn+ Macrophages. To distinguish between GvL and GvH reactivity, we performed comparative studies in either tumor-
Fig. 2  Immunohistochemical picture of frozen tissue sections that were triple-stained for CD8 (brown), CD4 (blue), and Sn (pink) in the livers of tumor-bearing mice (GvL) at day 5 (A and B) and day 20 (D and E) after ADI therapy and in the livers of non-tumor-bearing mice (GvH) at day 5 (C) and day 20 (F) after ADI. Magnification, ×100 (A and D) and ×200 (B, C, E, and F). pv, portal vein.

bearing mice (the GvL situation) or non-tumor-bearing mice (the GvH situation) after immune cell transfer. At different times after i.v. immune cell injection, the livers of the recipients were removed and snap-frozen to be analyzed later by immunohistochemistry. The number of detected T cells was calculated as the mean cell count ± SD obtained per section of a liver lobule. Triple staining with mAbs against CD4 and CD8 molecules for T cells and with mAb against Sn for Sn+ macrophages revealed the location of these cells in the liver sections of mice undergoing ADI therapy (10). We observed that these three types of cells formed tight clusters in which different numbers of each cell type were present. We define cell agglomerates containing at least one cell of each of the cell types described above as a cluster. Fig. 2 shows a representative
image of cluster formation in the early (day 5) and late (day 20) stages after immune cell transfer in GvL (A–C) and GvH (D–F). The clusters started to appear in the liver 5 days after donor cell transfer. They were located in the perportal areas close to the blood vessels and in the parenchyma. Fig. 3 shows the kinetics of cluster formation comparing the GvL and GvH situations. In the early stages (day 5) in the GvL situation, clusters were less abundant than in the later stages (day 20), when around 10 clusters/liver lobule could be found. In GvH, clusters contained smaller numbers of cells in both the early and late stages, and they disappeared from the liver around day 20. The numbers of cells within the clusters also varied (Fig. 2) and were highest in late-stage GvL. To elucidate cell surface membrane-membrane interactions within the clusters, we performed transmission electron microscopy studies 8 days after the immune cell transfer. Fig. 4 shows a typical section through a Kupffer cell (K) with its characteristic electro-dense cytoplasm, variety of lysosomal structures, and inclusions in close membrane interaction with two lymphocytes (L). Arrows point to areas of membrane contact between Kupffer and lymphocyte cells. Because Sn+ macrophages hardly formed clusters with T lymphocytes, we conclude that this macrophage-lymphocyte interaction is a special property of Sn-expressing macrophages.

Cluster-associated Sn+ Macrophages Are Activated. To investigate whether cluster formation might be a reflection of an active antigen presentation process between Sn+ macrophages and CD4 and CD8 T cells, liver sections were double-stained after immune cell transfer with SER4 mAb directed against the Sn molecule on macrophages and with mAbs against B7.1 (CD80) and B7.2 (CD86), costimulatory molecules involved in antigen presentation. After ADI, a dramatic increase in the number and staining intensity of Sn+ liver macrophages was observed. Previously, we found that in ADI-treated groups, Sn+ macrophages represented about 90% of all Kupffer cells, whereas in normal livers, their proportion was around 50%. As shown in Fig. 5, more Sn+ macrophages expressed B7.1 molecules during GvL (A) than during GvH (B). In GvL, the maximum expression occurred at day 12, when 64% of Sn+ macrophages were B7.1+. This level of expression remained constant until the last time point tested (28 days). In GvH, the maximum number of Sn+ B7.1+ cells appeared at day 28, with 63% of Sn+ macrophages being B7.1+. Interestingly, the number of Sn+ macrophages expressing the B7.2 molecule was very low in both the GvL and GvH situations. To investigate whether the increase in the number of host Sn+ macrophages was due to proliferation or to the recruitment of monocytes/macrophages from the blood circulation, liver sections were double-stained with SER and Ki67, a marker for proliferating cells. No double positivity was found in either the GvL or GvH situations. This suggests that the increased number is not due to the proliferative expansion of preexisting cells, but to the recruitment of nonproliferating cells into the organ.

Cluster-associated T Cells Are Proliferating. The activation of CD4 and CD8 cells within the clusters was studied by staining with mAbs against proliferation marker Ki67. Fig. 6 shows the kinetics of total and proliferating CD4 and CD8 cells in the GvL and GvH situations. In GvL (Fig. 6A), the maximum number of CD4 cells was reached at day 20. At this time point, 94% of them were Ki67+. Afterwards, the number of CD4 cells and, in particular, the number of proliferating CD4 cells decreased quickly. The number of infiltrating CD8 T cells was lower but also showed a peak at day 20, suggesting that both CD4 and CD8 T cells contribute to the ADI effect in a synergistic manner (4). However, in contrast to the CD4 population, the majority of CD8 T cells did not express Ki67. In GvH (Fig. 6B), we observed lower numbers of CD4 and CD8 cells than in GvL, and most of the cells did not proliferate.

Superantigen-reacting Vβ6 T Cells Form Clusters with Sn+ Macrophages. To test whether Vβ6 donor T cells that become activated by the antitumor immunization of donor mice and produce Th1 cytokines (Table 1) might also infiltrate the liver upon ADI, double stainings were performed including mAbs against Vβ6. Fig. 7 shows the kinetics of Vβ6 CD4 or CD8 T cells in the GvL (A) and GvH (B) situations. In tumor-bearing mice (GvL), the level of Vβ6 T cell infiltration increased dramatically at day 5 after ADI and then decreased progressively until day 28. This increase was mostly due to high proliferative activity, as shown by the Ki67 staining. Most of these infiltrating and proliferating Vβ6 T cells were CD4+. There was an increase in Vβ6/CD4 T cells from 10% in donor cells before immune cell transfer to 25% in the host liver. No increase was observed in the Vβ6/CD8 T cells. In non-tumor-bearing mice, Vβ6 T cells increased only at day 5, and to a lesser extent than that in GvL. The same organs were triple-stained with mAbs against Vβ6, CD4, CD8, and Sn, and the amount of double-positive Vβ6/CD4 or Vβ6/CD8 T cells localized in clusters together with Sn+ macrophages was evaluated (Fig. 8). In both the GvL (A) and GvH (B) situation, the ratio of Vβ6/CD4:Vβ6/CD8 double-positive cells was 2:1. In GvL, only about 45% of Vβ6/CD4 cells formed clusters with
Sn+ macrophages, whereas all Vβ6/CD8 cells were within the clusters. In the GvH situation, the total number of Vβ6 T lymphocytes was lower than that in the GvL situation. The proportion of Vβ6/CD4 cells in clusters in the GvH situation was the same as that in the GvL situation, but Vβ6/CD8 cells in the GvH situation were found to be associated not only with Sn+ macrophages, as in the GvL situation, but also as single cells.

**Vβ6+ Cells Induce Tumor Cytostasis.** To further investigate the possible effect of Vβ6 T lymphocytes on the tumor cells in the host liver, we evaluated their tumor cytostatic activity in vitro. Vβ6 T cells were isolated by magnetic bead column fractionation from the spleens of B10.D2 mice that were primed or nonprimed with ESb-MP lymphoma cells. The purity of the isolated Vβ6 T cells was >85% (fluorescence-activated cell-sorting staining). ESb-MP cells were cocultured with either Vβ6+ or Vβ6− cells, and the rate of proliferation was measured by [3H]thymidine incorporation after 48 h. As shown in Fig. 9, the coculture of ESb-MP cells with Vβ6+ cells induced a decrease in tumor cell proliferation (25% [3H]thymidine uptake when confronted with primed Vβ6 T cells in comparison to controls). There was also a dramatic decrease in the rate of proliferation when tumor cells were cocultured with Vβ6− T cells from primed mice. Interestingly, a difference between Vβ6+ and Vβ6− T cells was seen when using nonprimed cells. The former were significantly more cytostatic than the latter.

**DISCUSSION**

The data presented in this study demonstrate the formation of complex cell clusters in the livers of tumor-bearing mice upon ADI, leading to effective GvL reactivity. The clusters are composed of macrophages expressing Sn and of CD4 and CD8 T cells. Previous results showed that Sn+ macrophages increased dramatically upon immune cell transfer into tumor-bearing mice (10). Double staining with anti-Sn mAb and mAb against donor-type β2 microglobulin showed that this macrophage subpopulation was of host origin (21). In the present study, we found that Sn+ macrophages formed tight clusters with both CD4 and CD8 donor T cells; thus, these clusters could be sites of T cell-macrophage and of CD4/CD8 immune T-cell interactions. Synergistic interaction between immune CD4 and CD8 donor T cells was previously shown to be a prerequisite for a successful ADI therapy leading to a complete tumor regression.
and eradication of metastasis (4). The close contact of T cells with Sn+ macrophages, which was demonstrated by electron microscopy, makes a functional cooperation of these two types of cells very likely; for instance, they could interact in tumor antigen presentation and recognition and in antitumor immune responses. In support of this assumption, previous in vitro results demonstrated that Sn+ macrophages are able to process exogenous TAA from metastases and present it via MHC class II to CD4 (10) and via MHC class I to CD8 immune donor T lymphocytes. During GvL in our model, Sn+ macrophages were found to express costimulatory molecules such as B7.1 and, to a lesser extent, B7.2. It is known that the expression of these two molecules is regulated differently, and that B7.2 expression tends to precede B7.1 expression during an immune response (22).

In this study, we also investigated the proliferation status of the donor T cells that infiltrated the liver, using double staining for T cell markers and Ki67 as a proliferation-associated nuclear marker (20). An interesting difference between the liver infiltrates of CD4 and CD8 T cells was noted: whereas CD4 T cells showed a high proliferative activity, CD8 T cells had a very low proliferative activity. Nevertheless, both T cell subpopulations increased in number during ADI, and this was reflected by the fact that the number of cells within the clusters varied and were highest in late-stage GvL. Whether there is a selective recruitment of CD8 T cells to the metastatic liver remains to be elucidated. Sn+ macrophages, which also increased in number during ADI, did not express Ki67; thus, the possibility of a proliferative expansion from a liver-derived precursor population is unlikely. The increase in

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**Fig. 5** Kinetics of the accumulation of activated Sn+ macrophages in livers of GvL (A) and GvH animals (B) under ADI. Frozen tissue sections were single-stained for Sn+ macrophages (□) or double-stained for Sn+/B7.1+ (○) or Sn+/B7.2+ (●) with the corresponding mAbs. Three to six lobules/liver were analyzed under the microscope. Data show the means and SDs from two experiments with two to three animals/time point. Data points without SD represent the mean values for which the SD was smaller than 1.

**Fig. 6** Proliferation of CD4 and CD8 T lymphocytes in the livers of tumor-bearing (A) and non-tumor-bearing mice (B) under ADI treatment. Frozen tissue sections were either single-stained for CD4+ (■) or CD8+ (□) or double-stained for CD4+/Ki67+ (○) or CD8+/Ki67+ (Δ) with the corresponding mAbs. Three to six lobules/liver were analyzed under the microscope. Data show the means and SDs from two experiments with two to three animals/time point. Data points without SD represent the mean values for which the SD was smaller than 1.
absolute number, staining intensity, and the percentage of these cells after ADI may be due to the recruitment of monocytes from the blood followed by differentiation into Sn+ macrophages in the environment of the metastatic liver upon immunotherapy. Their recruitment may be due to the chemotactic factors released from tumor cells (such as macrophage inflammatory protein 1α, which is expressed in these tumor cells at the mRNA level6) or from immune cells interacting with the metastatic tumor cells in the liver.

This hypothesis is supported by the finding that the number of Sn+ macrophages in the GvL situation was higher than in the GvH system.

The molecular basis of the observed cluster formation needs to be further investigated. One possibility is that the Sn molecules on the macrophages function as adhesion molecules, facilitating the anchoring of CD4 and CD8 T cells to their surface for cell-cell interactions. A similar role of adhesion molecules, such as leukocyte function-associated antigen 1, has been reported for DCs interacting with resting B cells as a prerequisite for antibody synthesis (23) and in the homotypic cluster formation of human adult T-cell leukemia as an initial and essential step for cytokine production, proliferation of human adult T-cell leukemia, and hypercalcemia in vivo (24). Clustering of DCs, Th cells, and histocompatible B cells also occurs during primary responses in vitro (25). Other immune processes, such as the generation of specific CTLs, take place in

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cell clusters composed of cytotoxic and several Th cell subsets collaborating on the surface of APCs (26). In this process, T-cell subsets may require simultaneous antigen recognition and membrane interactions with APCs. Such a mechanism is likely to occur in our model, because we observed intensive membrane-membrane contacts between APCs and T cells by electron microscopy. An attractive possibility is the recently published "dynamic model" (27) that proposes that the three cells types, CD4 T cells, CD8 T cells, and APCs such as DCs, need not interact simultaneously, but sequentially. The helper cell can first engage and condition the DC, which then becomes licensed to stimulate a T killer cell. The identity of other molecules in APC/T-cell adhesion remains to be elucidated. If APC/T-cell adhesion uses mechanisms similar to those involved in leukocyte/endothelium adhesion, we would predict the involvement of integrins, chemokines, and their receptors.

The effectiveness of the described ADI was evaluated by staining the metastatic livers after immune cell transfer with proliferation marker Ki67. Tumor proliferation is usually determined by the number of tumor cells found in mitosis. The high number of clusters of proliferating cells in the liver early after cell transfer (day 2) indicated the presence of metastatic foci before donor immune cells reached the liver (day 5; Ref. 20). The number of tumor foci decreased with time of therapy, and they were absent from organs at a later stage of treatment (Fig. 1). The complete eradication of late-stage macroscopic metastases by this ADI protocol was also demonstrated noninvasively in intact animals by \(^{1}H\) nuclear magnetic resonance microimaging with respiratory triggering (28). In addition, noninvasive \textit{in vivo} \(^{31}P\) nuclear magnetic resonance spectroscopy revealed an early significant change due to ADI in tumor tissue pH by day 5–6 after treatment (29). Whether the observed disappearance of immune donor spleen cells from the liver at the later time points is due to apoptosis induced by host cells involved in host-versus-graft reactivity is under investigation. Host-versus-graft reactivity is expected to recover in 5-Gy-treated animals in about 3 weeks. Preliminary data indicate that the production of NO increased dramatically at day 20 after ADI, coinciding with the disappearance of immune cells. Previous data show that NO induces apoptosis in both murine and human tumor cells (30, 31). NO could thus be involved in the apoptosis of immune donor cells after therapy as a mechanism of their clearance from the liver.

In previous studies, we reported on the extraordinary immunoresistance of B10.D2 mice to highly metastatic tumor lines such as ESb derived from the mouse strain DBA/2 (32, 33). We have recently found that when typed for MMTV proviruses in their genome, B10.D2 and other resistant recombinant inbred mouse strains showed a selective loss of a long terminal repeat hybridization band that corresponded to Mtv-7. An open reading frame in the long terminal repeat of Mtv-7 codes for vSAG7 (Mi\(^{s}\)), an autoantigen of DBA/2 that causes the deletion of superantigen-reactive T-lymphocytes such as V\(\beta\)_6+ cells during thymus maturation (34, 35). Suggestive evidence for a link between tumor resistance and the absence of endogenous vSAG was given by a back-cross segregation analysis as well by the reintroduction of the superantigen into the resistant line and the reappearance of tumor susceptibility (5). ESb tumor cells, as well as the syngeneic mouse strain DBA/2, express proviral Mtv-7 at the mRNA level. It is possible that after the injection of ESb tumor cells into B10.D2 tumor-resistant mice that do not express the superantigen Mtv-7, V\(\beta\)_6 superantigen-reactive T cells become activated and, after an adoptive transfer into ESb tumor-bearing DBA/2 mice, infiltrate ESb liver metastasis and recognize the superantigen. Other groups have reported that human V\(\beta\)_6 T cells recognize TAAs in ovarian cancer (36), gastric carcinomas (37), adrenal carcinomas (38), and neoplasms of the central nervous system (39). In our GvL mouse model, it may be important to find out how superantigen-reactive V\(\beta\)_6 T cells contribute to the therapy effect. Therefore, we double-stained metastatic livers with antibodies against V\(\beta\)_6, CD4, CD8, and Ki67 at different time points after therapy. During GvL, V\(\beta\)_6 T cells possessed a high proliferation rate and were found more in the CD4 T-cell population than in the CD8 T-cell population. In GvH, V\(\beta\)_6 T cells were almost absent. This supports the hypothesis of a reactivity of these cells against the tumor-associated superantigen. V\(\beta\)_6 T cells were also present in clusters in contact with Sn+ macrophages. Superantigens are known to bind to MHC class II molecules and to certain V\(\beta\) T-cell receptor chains, thus resulting in the cross-linking of both molecules and the subsequent activation of the T cell (40). In our model, the interaction of the superantigen and the MHC class II molecules in the Sn+ macrophages would need the cleavage of the superantigen from the surface of the tumor cells and then the transfer of the cleaved form to the MHC class II molecules on the Sn+ macrophages. vSAGs contain furin-like cleavage sites and are naturally cleaved. In particular, vSAG7 is synthesized as a Mr 45,000 transmembrane glycoprotein precursor but is proteolytically processed to yield a Mr 18,500 surface protein that is the functional form of the superantigen (41). Second, the interaction of this functional form of the superantigen with the Sn macrophages might happen by paracrine transfer as described previously (11) in a process that requires not cell-cell contact but cellular proximity. Additional experi-

![Fig. 9](image-url) Influence of V\(\beta\)_6 T cells on the proliferation of ESb-MP tumor cells in vitro. Tumor cells were cultured for 48 h alone ( ), in combination with nonprimed ( ) and ESb-MP-primed ( ) B10.D2-derived V\(\beta\)_6– T cells or nonprimed ( ) and primed ( ) V\(\beta\)_6 + T lymphocytes, respectively. The proliferation rate of ESb-MP tumor cells was measured in three independent experiments using the \(^{3}H\)thymidine assay. Data are expressed as a percentage of the proliferation rate of tumor cells cultured alone (control). Bar. means ± SD.
ments are required in our model to find out the mechanism of recognition, binding, processing, and presentation of the tumor-associated Mtv-7 superantigen.

To test whether Vβ6 T cells could have a direct effect on tumor cells, we performed an in vitro assay in which immuno-~purified Vβ6 T cells from primed and nonprimed mice were cocultivated with ESb-MP tumor cells. The data presented show a direct cytostatic effect of Vβ6 T cells on ESb-MP tumor cells. Interestingly, the effect was very similar in Vβ6 T cells from primed and nonprimed mice. In contrast, only primed Vβ6− cells produced a strong antitumor cytostatic effect. One may speculate that other cells besides the Vβ6 T cells are also involved in the observed therapeutic effect and need to be primed before the therapy to recognize either minor histocompatibility antigens or the TAA. This would suggest that in vivo, more than one T-cell-mediated effector mechanism (SAG-reactive and TAA-reactive cells, CD4 and CD8 T cells) needs to be operative, and that cooperation between T cells must be established to achieve the observed complete tumor regression of late metastases. Vβ6− T cells recognizing minor histocompatibility antigens and TAs would need to be primed before transfer, but Vβ6+ cells recognizing the superantigen directly would not require priming. We also provide evidence that donor Vβ6 T cells are activated after tumor cell injection and produce high amounts of IL-2 and IFN-γ just before cell transfer. This activation and cytokine production may be particularly important for other subpopulations of donor T lymphocytes. We suggest that Vβ6 donor T cells are able to mediate antitumor activity after recognition of the Mtv-7 superantigen on the surface of ESb tumor cells and in sites of liver metastases during ADI in this GvL model.

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