Successful Treatment of Canine Malignant Histiocytosis with the Human Major Histocompatibility Complex Nonrestricted Cytotoxic T-Cell Line TALL-104

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ABSTRACT

The human MHC nonrestricted cytotoxic T-cell line TALL-104 exerts potent antitumor effects in animal models with both induced and spontaneous cancers. The present report documents the ability of systemically delivered TALL-104 cells to induce durable clinical remissions in four of four dogs with malignant histiocytosis (MH). The animals received multiple i.v. injections of lethally irradiated (40 Gy) TALL-104 cells at a dose of 10⁶ cells/kg, with (two dogs) or without (two dogs) cyclosporin A, followed by monthly boosts. No significant clinical or laboratory toxicities developed during cell therapy; interestingly, a strong correlation was found between the dogs’ clinical and immunological responses. One dog with advanced disease (intrathoracic involvement) refractory to chemotherapy achieved a complete remission (CR) within 2 months of the first TALL-104 cell infusion. This dog died 14 months later of unrelated disease. The other three dogs with MH that at diagnosis had multiple s.c. and cutaneous lesions and lymphadenopathy, but no visceral involvement, were treated with TALL-104 cells as single agent (no chemotherapy was administered). Two of these dogs achieved a CR soon after cell therapy, and the third dog had two long-lasting partial responses; CR in this dog was later achieved by combined administration of chemotherapy and cell therapy. None of the three dogs that received cell therapy at diagnosis developed visceral disease in the ~9–22 months of follow-up. The clinical responses experienced by all four MH cases to TALL-104 cell therapy suggest the high responsiveness of this canine tumor to these xenogenic effectors and their therapeutic potential even in the most aggressive forms of the disease.

INTRODUCTION

MH in dogs, first reported in 1978 (1), is a tumor characterized by neoplastic proliferation of invasive atypical erythropagocytic histiocytes in various tissues. The disease frequently becomes manifest in the middle-age years and has been observed more frequently in males than in females (2). Bernese mountain dogs are genetically prone to this type of cancer (3, 4), but other breeds are also sporadically affected (5). Clinical findings commonly include fever, generalized lymphadenopathy, and hepatosplenomegaly as well as concomitant anemia, leukopenia, and thrombocytopenia (1). Neoplastic histiocytes mainly infiltrate the spleen, liver, lungs, lymph nodes, bone marrow, and skin. The visceral form of this disease is rapidly progressive, and the prognosis is poor (1). The diagnosis of MH presents a challenge to both clinicians and pathologists because the disease lies within a spectrum of histiocytic and hematolymphoid disorders and may be confused with granulomatous inflammation. Recently, phenotypic similarities between malignant fibrous histiocytoma and malignant histiocytosis have been reported (6). A diagnosis of MH is appropriate once the malignant nature and histiocytic differentiation of the process have been established.

We have developed a new cell therapy approach based on the use of the human leukemic T-cell line TALL-104 (CD3/TCRαβ⁺, CD8⁺, CD56⁺, and CD16⁺), which is endowed with potent MHC nonrestricted tumoricidal activity, without lysing cells from normal tissues (7, 8). Unlike patient-derived lymphokine-activated killer cells, TALL-104 cells provide an unlimited and reliable source of clonal effector cells with stable cytotoxic activity that is ideal for cell therapy approaches. Although dependent on recombinant human IL-2 for expression of cytotoxicity and long-term survival in vitro, TALL-104 cells can exert antitumor effects in vivo without the concomitant administration of IL-2, thus eliminating the potential toxicity of this cytokine. TALL-104 cells induce necrotic tumor cell death through secretory pathways involving perforin and serine esterases and/or kill targets through the release of cytostatic/cytotoxic mediators such as TNF-α, TNF-β, IFN-γ, or transforming growth factor β (7, 9). Despite the fact that TALL-104 cells would be rejected by a tumor-bearing MHC-incompatible host.

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The abbreviations used are: MH, malignant histiocytosis; CsA, cyclosporin A; PBMC, peripheral blood mononuclear cell; FACS, fluorescence-activated cell-sorting; TNF, tumor necrosis factor; GM-CSF, granulocyte macrophage-colony stimulating factor; CR, complete response; PR, partial response; IL, interleukin; NK, natural killer.
we have used γ-irradiation (40 Gy) as a precautionary measure to irreversibly arrest the growth of this leukemic cell line in the host tissues (10). This treatment did not impair the antitumor effects of TALL-104 cells in immunodeficient mice engrafed with human neoplasms (11, 12) and in immunocompetent mice bearing syngeneic leukemia (13), suggesting the feasibility of using γ-irradiated (nonproliferating) TALL-104 cells in cancer therapy.

To determine the potential safety of TALL-104 cells as an antitumor agent in a clinically relevant setting, we recently conducted a Phase I trial in pet dogs with spontaneous end-stage tumors (14). Results of that study clearly showed not only the absence of significant adverse reactions in terminally ill canine patients but also the manifestation of various levels of clinical responses including one CR and one PR in two dogs with MH. The present report extends the follow-up for these two dogs and describes the successful application of TALL-104 cell therapy to two more dogs with less-advanced MH. Results indicated that: (a) this potentially fatal canine disease is extremely sensitive to treatment with xenogeneic TALL-104 cells; (b) durable CRs could be achieved even in dogs with advanced tumors, refractory to chemotherapy; and (c) TALL-104 cells were very effective when used as a single agent in newly diagnosed cases with systemic disease but no visceral involvement.

**MATERIALS AND METHODS**

**Canine Patients.** Four dogs diagnosed with MH were treated with TALL-104 cells at the Veterinary Oncology Services and Research Center (West Chester, PA), starting in November 1994 (Tables 1 and 2). Complete work-up, including complete blood counts, kidney and liver functions, chest X-rays, abdominal ultrasounds, and bone marrow aspirate, was performed at diagnosis. The clinical characteristics of these patients at the beginning of cell therapy are summarized in Table 1. Dog 1 was enrolled in the study in 1994; at the time of diagnosis, he had advanced disease with skin, lymph nodes, and intrathoracic involvement (14). Although this dog had originally responded to chemotherapy with a CR, progressive disease developed, and decreased cardiac contractility precluded further treatment with doxorubicin. The other three dogs (dogs 2, 3, and 4) that entered the TALL-104 cell study in 1995 and 1996 were newly diagnosed cases with disease limited to skin and lymph nodes; because of their less advanced stage and because of the dramatic response to TALL-104 cell therapy seen in dog 1 (14), these three dogs were not given chemotherapy and were treated with TALL-104 cells as the single agent.

**Histological and Immunohistochemical Analysis.** Sections (6 μm) of paraffin blocks from the dogs’ tumor lesions were stained with H&E for histological analysis. Immunohistochemical staining was done using the avidin-biotin-peroxidase complex method (15): sections were deparaffinized, dehydrated through graded alcohol, and washed in 0.01 M PBS (pH 7.4). To block nonspecific binding, sections were incubated with normal horse serum (1:75; Vector Laboratories, Burlingame, CA) for 20 min, followed by overnight incubation at 4°C with primary antibodies antilysozyme (1:200), anti-α-1-antitrypsin (1:300; DAKO, Carpinteria, CA), and antitissue transglutaminase (1:300; ICN Biochemicals, Costa Mesa, CA). Normal mouse serum was used as control antibody. Sections were incubated in biotinylated horse antimouse IgG (1:200; Vector Laboratories) for 45 min. All incubations were done in a humidified chamber at room temperature. 3-Amino-9-ethylcarbazole (Sigma Chemical Co., St. Louis, MO) was used as chromogen, and sections were counterstained with Mayer’s hematoxylin.

**Large-Scale Expansion of TALL-104 Cells for Therapy.** TALL-104 cells were grown in endotoxin-free Iscove’s modified Dulbecco’s medium (Life Technologies, Inc., Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (Atlanta Biologicals, Norcross, GA) and 100 units/ml recombinant human IL-2 (Chiron Therapeutics, Emeryville, CA) in a humidified incubator at 37°C with 10% CO2 in T-175 vented cap flasks (Falcon, Franklin Lakes, NJ). Mycoplasma contamination was monitored weekly on cell samples from 10 randomly chosen flasks using a commercial PCR kit (ATCC, Rockville, MD). At the time of therapy, cells were harvested, centrifuged in conical centrifuge tubes (Corning, New York, NY), washed twice in saline (Abbott Laboratories, King of Prussia, PA), resuspended in 100 ml of saline, γ-irradiated (40 Gy) using a 137Cs source, and transferred to a blood transfusion pack (Baxter Diagnostics, Inc., Glendale, PA) for systemic administration within 2-4 h of irradiation. Cell aliquots were removed from the bag for determination of cytotoxic activity and sterility (quality control assays).

**Protocols of TALL-104 Cell Administration.** Table 2 summarizes the schedule of TALL-104 cell infusions received by each dog in the study. Cells were γ-irradiated (40 Gy) and administered at a dose of 107/kg in saline as an i.v. bolus over 30 min. Cell boosts were given at the indicated intervals and

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**Table 1 Characteristics of the MH dogs treated with TALL-104 cell therapy**

<table>
<thead>
<tr>
<th>Canine patient</th>
<th>Sex/age/yr/breed</th>
<th>Previous therapy</th>
<th>Clinical stage at diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dog 1</td>
<td>M/8/Scottish Terrier</td>
<td>Five cycles of chemotherapy with doxorubicin, cyclophosphamide, vincristine, dacarbazine, and l-asparaginase</td>
<td>Multiple cutaneous and s.c. lesions, metastatic lymphadenopathy, intrathoracic involvement</td>
</tr>
<tr>
<td>Dog 2</td>
<td>M/7/West Highland Terrier</td>
<td>None</td>
<td>Multiple cutaneous and s.c. lesions, metastatic lymphadenopathy</td>
</tr>
<tr>
<td>Dog 3</td>
<td>F/3/Bernese Mountain Dog</td>
<td>None</td>
<td>Cutaneous lesions on the muzzle and right axilla, metastatic lymphadenopathy</td>
</tr>
<tr>
<td>Dog 4</td>
<td>F/1/Golden Retriever</td>
<td>None</td>
<td>Cutaneous and s.c. lesions on the muzzle; metastatic lymphadenopathy</td>
</tr>
</tbody>
</table>

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performed on serum samples and PBMCs (prespudy, 7.8 pg/ml for TNF-α, 8 pg/ml for TNF-β, and 7.8 pg/ml for GM-CSF (14).

Cytokine-specific ELISA kits (Endogen, Boston, MA) according to the manufacturer’s instructions. The sensitivity of the assay was 20 pg/ml for IFN-γ and TNF-α, 8 pg/ml for TNF-β, and 7.8 pg/ml for GM-CSF (14).

RESULTS

Diagnosis of MH. The diagnosis of MH in all four dogs was made based on clinical and morphological criteria (histology reports from veterinary pathologists) and confirmation of the histiocytic lineage of tumor cells by immunohistochemical staining (positive staining for the histiocytic markers α-1-trypsin, cathepsin B, and lysozyme; data not shown).

Clinical Responses to TALL-104 Cell Therapy. Table 3 summarizes the clinical responses observed in the four dogs with MH to TALL-104 cell therapy (updated to June 1997). At the time of initiation of cell therapy, dog 1 had prefemoral and left inguinal enlarged lymph nodes and right axillary lymphadenopathy, and its performance status was poor (modified Karnofsky performance status = 2). After 2 weeks of systemic TALL-104 cell therapy (six injections), the right axillary node completely regressed, but the other nodes remained unchanged. Two cell boosts, 1 week apart (see Table 2), were then admin-

Clinical Responses to TALL-104 Cell Therapy. The presence of human IFN-γ, TNF-α, GM-CSF, and TNF-β in serum samples collected presudy and throughout cell therapy was tested using human cytokine-specific ELISA kits (Endogen, Boston, MA) according to the manufacturer’s instructions. The sensitivity of the assay was 20 pg/ml for IFN-γ and TNF-α, 8 pg/ml for TNF-β, and 7.8 pg/ml for GM-CSF (14).

Immunological Monitoring. Immunological studies were performed on serum samples and PBMCs (prespudy, be-

To monitor the development of TALL-104-specific cellular immune response, PBMC samples were tested against 51Cr-labeled TALL-104 cells in an 18-h 51Cr-release assay, as described above.

PCR Analysis. PBMC samples from the dogs were obtained immediately before each TALL-104 cell infusion and at various intervals during and after cell therapy, as indicated in the "Results”; DNA was extracted from the PBMCs and frozen (14). The presence of circulating TALL-104 cells in each cell extract was evaluated by PCR analysis using two primers specific for the human minisatellite region YNZ.22 (16). An oligonucleotide probe recognizing 24 nucleotides in the middle of the amplified sequence was used to demonstrate the specificity of the PCR products by Southern blot hybridization, as described previously (16).

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Table 2 Protocols of TALL-104 cell administration to dogs with MH

<table>
<thead>
<tr>
<th>Canine patient</th>
<th>Date of cell therapy initiation</th>
<th>Schedule</th>
<th>Number of boosts</th>
<th>CsA (10 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dog 1</td>
<td>11/28/94</td>
<td>Every other day for 2 weeks</td>
<td>2 (weekly)</td>
<td>Yes</td>
</tr>
<tr>
<td>Dog 2</td>
<td>8/21/95</td>
<td>Daily for 5 days, 2-week rest, daily for 5 days</td>
<td>20 (monthly)</td>
<td>Yes</td>
</tr>
<tr>
<td>Dog 3</td>
<td>1/16/96</td>
<td>Daily for 5 days</td>
<td>15 (monthly)</td>
<td>No</td>
</tr>
<tr>
<td>Dog 4</td>
<td>9/1/96</td>
<td>Daily for 5 days, weekend rest, daily for 5 days</td>
<td>8 (monthly)</td>
<td>No</td>
</tr>
</tbody>
</table>

Toxicity Monitoring. Clinical signs of acute toxicity (such as fever, chills, hypotension, diarrhea, and vomiting) were monitored during and after each cell injection. All dogs were treated as outpatients during the course of TALL-104 cell therapy, and the owners were properly instructed to report on the well-being of their pets during therapy. Blood and serum samples (for complete blood counts, serum chemistry, cytotoxicity assays, cytokine production, and immunological monitoring; see below) were obtained from the patients before the study, immediately before each TALL-104 cell administration, and throughout the follow-up period.

Cytotoxicity Assays. PBMCs were isolated from heparinized blood samples by Accu-Prep (specific gravity, 1077 g/ml; Accurate Chemical, Westbury, NY) lymphocyte gradient centrifugation and stained as effectors in an 18-h 51Cr-release assay against the human leukemic target cell lines U937 and K562 (both NK-sensitive) and TALL-106 (NK-resistant). This test was routinely used to monitor the possible development of MHC nonrestricted cellular immune responses against human (xenogeneic) cells. Briefly, a fixed number of 51Cr-labeled target cells (10^6 cells/well) were tested against four effector cell lines of cells administered for 5 consecutive days (Table 2).

Clinical Responses to TALL-104 Cell Therapy. Table 2 summarizes the protocols of TALL-104 cell administration to dogs with MH.

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</tr>
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</table>
cell therapy of canine malignant histiocytosis

Table 3: Clinical responses to TALL-104 cell treatment in dogs with MH

<table>
<thead>
<tr>
<th>Canine patient</th>
<th>Clinical response</th>
<th>Duration (mo)</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dog 1</td>
<td>CR</td>
<td>14</td>
<td>Died of causes unrelated to cancer</td>
</tr>
<tr>
<td>Dog 2</td>
<td>First PR</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Second PR</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CR</td>
<td>4+</td>
<td></td>
</tr>
<tr>
<td>Dog 3</td>
<td>CR</td>
<td>16+</td>
<td>NED</td>
</tr>
<tr>
<td>Dog 4</td>
<td>CR</td>
<td>9+</td>
<td>NED</td>
</tr>
</tbody>
</table>

* The CR in dog 2 was achieved by a combination of chemotherapy/cell therapy.

* NED. no evidence of disease.

istered; during this period of time, a significant improvement in the clinical well-being of dog 1 was documented (modified Karnovsky performance status = 0), with complete regression of the axillary lymph node and >50% decrease in the inguinal lymph nodes and in the neck mass. At this time, a thoracic radiograph was negative for lymphadenopathy and pulmonary infiltrates. TALL-104 cell therapy was ended at this point. At reexamination 1 week later, new s.c. nodules were found on the dog’s left hind and left front legs. The nodules were confirmed to be a recurrence by cytological examination of needle aspirates. Surgical removal of the nodules followed by in vitro analysis of the susceptibility to TALL-104 cell lysis was not possible, because 2 days later, the lesions had regressed by >50%. At reexamination 2 weeks later, no nodular lesions were palpable, and no signs of disease were detected. A state of long-lasting CR was achieved, documented by the absence of clinical signs of relapse on repeated abdominal ultrasounds and thoracic X-rays throughout the rest of the dog’s life (14 months). Dog 1 developed recurrent bacterial cystitis approximately 6 months after its CR. A mild azotemia subsequently developed, and abdominal ultrasound showed changes consistent with kidney pyelonephritis. The disease was stabilized for a few months using antibiotics and a low-protein diet as supportive therapy; however, renal failure grew more severe, and dog 1 eventually died with a clinical picture of acute abdominal disease with ascites and bloody diarrhea. Necropsy revealed ischemic necrosis of the intestine (ultimate cause of death), but no macroscopic signs of MH. In particular, its spleen and liver, which are the classic sites of systemic MH (1–5), seemed normal. Histological analysis (H&E staining) was performed blindly by pathologists at the University of Pennsylvania Veterinary School of Medicine on formalin-fixed, paraffin-embedded necropsy samples of the dog’s kidneys, bladder, liver, spleen, stomach, intestine, heart, and lungs and of the mesenteric and mediastinal lymph nodes. No infiltrating malignant histiocytes were identified in any of these organs, and no evidence of other types of cancer was found at necropsy. On the other hand, severe chronic lymphoplasmacytic tubulointerstitial nephritis was found together with other organ changes, such as uremic pneumonia and uremic gastritis (data not shown), typically seen in severe kidney failure leading to uremia. Moreover, signs of thromboembolism were detected in the liver and spleen and around the abdominal lymph nodes (not shown), consistent with a pathological diagnosis of thromboembolic disease, which has been described to be associated with renal failure (17, 18).

Dog 2 was the second dog with MH treated with TALL-104 cells (and CsA; Table 3). This dog also had participated in our Phase I study (14). Before cell therapy, the dog had multiple cutaneous nodules between pads and along the metatarses of the right hind leg with metastatic popliteal lymph node. Within 3 weeks from the first 5-day cycle of TALL-104 cells, the most proximal cutaneous lesions completely regressed, whereas the more distal mass had only partially regressed, and the popliteal lymph node had remained unchanged. A second 5-day cell cycle was then administered. In the following month, dog 2 achieved a PR (>50% reduction of cutaneous masses and popliteal lymph node) that lasted for 2 months (Table 3). After the second month, both the interdigital masses and lymph node grew back. A second PR was achieved after 2 monthly boostings with TALL-104 cells and lasted about 6 months with stabilization of the cutaneous lesions. No signs of visceral disease were seen in this dog throughout cell therapy. When the lesions of the right hind leg started to progress again (12 months from start of cell therapy), the decision was made to suspend TALL-104 cell treatment. Chemotherapy (doxorubicin and dacarbazine) given to this dog for 5 days induced a CR of the skin lesions and a PR of the popliteal lymph node. Aspiration cytology of the lymph node did not reveal any evidence of intact histiocytes. Another 5-day TALL-104 cell cycle, given 2 weeks later as maintenance therapy, resulted in a CR with complete disappearance of the lesions. At the time of this writing, dog 2 has no evidence of cutaneous or visceral disease (4+ months; Table 3).

Dog 3 was diagnosed with MH in December 1995 and started TALL-104 cell treatment in the middle of January 1996.
Fig. 2  Cytotoxic activity of PBMCs from three dogs with MH on TALL-104 therapy. PBMC samples were harvested at the indicated times (d, day; w, week) after initiation of cell therapy and tested for killer activity against K562 (☐) and TALL-104 (○) cells in an 18-h $^{51}$Cr release assay. The E:T ratio was 50:1. Arrows in the Dog #2 panel indicate the times of relapse, documented clinically.

(Table 2). At the time of diagnosis, the dog had cutaneous lesions on the muzzle and right axilla, which were histologically confirmed to be cutaneous histiocytosis with a biopsy. There were also regional metastatic lymph nodes. Chest X-rays and abdominal ultrasound excluded intrathoracic and abdominal involvement, respectively. One month after one 5-day TALL-104 cell cycle, a decrease in the cutaneous lesions and axillary lymph nodes started to be noticeable. After the first 2-day boost with TALL-104 cells, the right axillary lymphadenopathy completely regressed, and the cutaneous lesions went down by more than 50% of their initial size. However, 1 month later, just before the second cell boosting was scheduled, new cutaneous lesions appeared along the muzzle. The dog received his second 2-day cell boost, and in the following month, the new cutaneous lesions completely regressed: a long-lasting CR was then achieved with no signs of cutaneous or visceral disease recurrence, as documented in follow-up visits. Dog 3 remains disease-free at the time of preparation of this manuscript (16+ months; Table 3).

Dog 4 was immediately treated with TALL-104 cells at diagnosis in September 1996. At this time, the dog had multiple
Fig. 3 Humoral immune responses against TALL-104 cells mounted by the four dogs with MH on cell therapy. Sera of the treated dogs were harvested at the indicated times (d, day; w, week; m, month) after initiation of cell therapy, diluted at 10^{-3}, and tested for the presence of TALL-104-specific antibodies. The percentage of reactivity with TALL-104 cells stands for the percentage of TALL-104 cells binding immunoglobulin in the canine sera, as tested by FACS analysis.

Clinical Laboratory Changes and Toxicity Associated with TALL-104 Cell Therapy. Dogs 1 and 2 exhibited vomiting (responsive to antiemetics) and diarrhea (treated with diphenoxylate/atropine) during cell administration and in the following 24 h. These symptoms could be prevented in dog 2 by premedication with an antihistaminic. The other two dogs did not show any signs of clinical toxicity associated with TALL-104 cell injections. Blood chemscreens, run regularly on sam-
and at different times after TALL-104 cell injections. In all gels: Lane 1, TALL-104 cells (positive control) and Lane 2, water (negative control). In the gel for dog 1: Lane 3, pre-TALL-104 cell therapy; Lanes 4 and 5, days 3 and 5, respectively, of the first TALL-104 cell therapy cycle; Lanes 6–8, 1, 2, and 3 weeks after the last TALL-104 cell cycle. In the gel for dog 2: Lanes 3 and 4, days 3 and 5 of the first TALL-104 cell cycle; Lanes 5 and 6, 12 and 36 days after the first TALL-104 cell injection; Lane 7, day 2 of the first TALL-104 cell boost; Lanes 8 and 9, 6 and 9 months later; Lane 10, day 2 of the 10th TALL-104 cell boost; Lane 11, 1 year after beginning of TALL-104 cell therapy. In the gel for dog 3: Lanes 3–6, 5, 6, and 7 months after the beginning of TALL-104 cell therapy; Lane 7, day 2 of the TALL-104 cell boost given 8 months after beginning of cell therapy; Lane 8, ninth boost. In the gel for dog 4: Lane 3, pre-TALL-104 therapy; Lane 4, day 4 of the 5-day TALL-104 cell therapy cycle; Lanes 5–8, days 11, 18, 22, and 28 after the first TALL-104 cell injection. PCR assays on samples from dogs 1 and 2 were run at different times (with new controls each time). PCR assays on the samples from dogs 3 and 4 were performed at the same time using cryopreserved PBMCs.

In the case of dog 2, the correlation between cell-mediated immunity and disease progression/regression was even more evident because of the fluctuating course of his disease and the closer monitoring of his immunity (Fig. 2); three peaks of cytotoxic activity by dog 2’s PBMCs against K562 cells were detected, the first during the initial PR, the second at the time of the first relapse and during the second PR, and the third at the time of the second relapse. In the periods of time between tumor regression (when the disease was stable), the cytotoxic activity of this dog’s PBMCs was close to 0%. Similar curves were seen in the case of dog 3 and dog 4 (Fig. 2), although their clinical courses were much simpler (CR without clinically detectable relapses).

**Immune Response Against TALL-104 Cells.** No cellular immune responses against TALL-104 cells could be demonstrated in PBMC samples from dog 1 at any interval during and after cell therapy (data not shown), whereas specific CTLs against TALL-104 cells were present in the PBMCs of the other three dogs (Fig. 2). Despite the immunosuppressive regimen with CsA, dogs 1 and 2 developed antibodies against TALL-104 cells between the second and third week of treatment (Fig. 3). The same was true for dog 3 and dog 4 that did not receive any immunosuppressive drug (Fig. 3). This humoral immune response in dogs 1, 2, and 3 was still present 1 year after beginning cell therapy (Fig. 3). However, none of the TALL-104-reactive serum samples tested was able to inhibit TALL-104 cell tumoricidal activity, as determined by in vitro cytotoxicity assays against human tumor target cell lines (data not shown).

**Detection of Circulating TALL-104 Cells.** PCR amplification of the human minisatellite region YNZ-22 performed on the PBMCs of the TALL-104-treated dogs documented the presence of TALL-104 cells in the circulation at various time points during therapy as well as their disappearance within 5 days after each injection (Fig. 4). The lack of long-term persistence of TALL-104 cells in the circulation was confirmed by PCR analysis in each dog months after the beginning of cell therapy (Fig. 4).

**DISCUSSION**

Since its establishment from a child with T-cell leukemia (7), the TALL-104 cell line has been characterized extensively both in vitro, for its ability to selectively recognize and kill tumor cells in a MHC nonrestricted fashion, and in vivo, for its antitumor efficacy in animal models with transplantable malignancies. Although the antigenic structures recognized by TALL-104 cells on the surface of tumor cells remain to be defined, such structures seem to be phylogenetically well conserved, because TALL-104 cells can kill tumors across species. Moreover, normal cells seem to escape TALL-104 cell killing or...
damage (8, 10). The lack of TALL-104 cell toxicity to healthy tissues has been shown in a variety of animal models, including mice, dogs, and nonhuman primates (20). The possibility of administering TALL-104 cells as a single antitumor agent represents an advantage over the use of the patient’s own lymphokine-activated killer cells, which requires the concomitant infusion of toxic doses of IL-2 for efficacy (21). In addition, the continuous availability of TALL-104 cells, the possibility of expanding them to therapeutic doses (10⁸ cells/kg) for repeated infusions, their high migratory activity to tumor sites (22), their antitumor efficacy even in the absence of cell proliferation (after lethal irradiation), and their ability to induce either necrotic or apoptotic death in tumors across MHC barriers (7-14) are all aspects pointing to the feasibility, safety, and efficacy of these cells as an anticancer agent. The Phase I preclinical trial we recently conducted in canine patients with advanced tumors (14), which included dog 1 and dog 2 of the present report, has revealed important information on the possible use of TALL-104 cells in a clinical setting, specifically their ability to antagonize tumor growth even in an advanced disease setting of refractory malignancies, without inducing significant adverse reactions.

The findings in the present report are particularly encouraging, considering the aggressiveness and the poor prognosis of MH in most of the dogs and in humans. In this context, the achievement of a durable CR in a dog with a very advanced disease (dog 1) on systemic TALL-104 cell infusion was unexpected, as was the regression of its relapsed nodules after the completion of cell therapy. The latter observation suggested the development of a state of specific antitumor immunity that protected this dog from further relapses throughout its life span. Immunological studies of this dog’s blood samples before, during, and after cell therapy showed the appearance of MHC nonrestricted cell-mediated cytotoxicity against human tumor cells (both NK-sensitive and -resistant) during TALL-104 therapy. with a peak response at the time of regression of the relapsed nodules. The same pattern of responses was seen in the other three dogs treated. Unfortunately, no tumor specimens were available from the dogs during this study to test the development of specific antitumor immunity. However, experiments with other dogs in our Phase I trial (14) demonstrated the ability of their PBMCs to recognize and kill their own tumor cells in vitro during the course of TALL-104 cell therapy. The induction of endogenous antitumor immunity on passive transfer of TALL-104 cells was recently demonstrated in a murine model of syngeneic leukemia in which TALL-104-treated and cured mice were able to reject additional leukemia cell challenges (13). That the tumor regression observed in dogs 1, 2, and 4 was a consequence of TALL-104 cell treatment was suggested by the facts that: (a) the disease in dog 1 was progressing under chemotherapy; and (b) none of these three dogs received other drugs (aside from CsA) during and/or after completion of cell therapy. It is also unusual that the disease in dog 2 did not systemically progress 22 months after the diagnosis. Moreover, to our knowledge, no spontaneous remissions have ever been documented in dogs with MH; therefore, the tumor regressions achieved upon single-agent TALL-104 cell therapy are to be considered significant.

Because of the novelty of infusing human (xenogeneic) effector cells for treatment of canine malignancies, it was crucial in the present study to demonstrate not only the lack of acute toxicity in these animals as an immediate consequence of TALL-104 cell infusion (± CsA), but also the lack of chronic tissue injury resulting from the release of toxic cytokines and/or to a slow clearance of dying TALL-104 cells. Although laboratory findings showed no direct evidence of acute kidney toxicity during the course of cell therapy, the development in dog 1 of severe chronic pyelonephritis a few months after systemic injection of TALL-104 cells raises the question of a causal relationship between CsA/cell therapy and the onset of nephritis. In this regard, dog 1 had recurrent episodes of bacterial cystitis before the onset of evident renal disease, with no alterations in kidney functions detectable during cell treatment. Kidney abnormalities were also absent in all long-term survivors enrolled in our Phase I clinical trial with TALL-104 cells with or without CsA (14) and in the other dogs with MH described in this study. In addition, no significant changes in kidney function were observed in long-term toxicity studies performed on healthy dogs and nonhuman primates that received multiple systemic TALL-104 cell injections (20). On the other hand, a high frequency of renal disease has been reported in a number of terrier breeds (23, 24).

The histopathological changes noted on dog 1’s kidneys at the time of postmortem examination were not compatible with those found with CsA-associated nephrotoxicity or with autoimmune nephropathy. Renal biopsies of CsA-treated patients show an interstitial fibrosis with tubular atrophy (25). Toxic tubulopathy, peritubular capillary congestion, arteriopathy, and a striped form of interstitial fibrosis with tubular atrophy may also be present (25). On the other hand, immunologically induced nephropathy is usually characterized by primary alterations of the glomeruli (membrane-proliferative glomerulonephritis; Ref. 26) that were absent in dog 1’s kidneys. The remarkably long-lasting clinical responses associated with activation of the immune system seen in this study on TALL-104 cell therapy in dogs with histologically documented MH suggests the potential of these cells for treatment of this malignancy. To fully explore the feasibility of the TALL-104 cell approach in an adjuvant setting, we are now conducting a Phase II/III clinical trial in dogs with minimal disease after remission induction for different types of cancer. These studies will be crucial to the identification of tumors that are either resistant or highly susceptible to TALL-104 therapy and, ultimately, to the development of optimal treatment protocols against both sets of tumors. These protocols envision the application of TALL-104 cells as either a single agent or in combination with chemotherapy, depending on the clinical responsiveness of the tumor being treated.

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