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Toxicological and immunological evaluation of the MHC-non-restricted cytotoxic T cell line TALL-104

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Abstract The human MHC-non-restricted cytotoxic T cell line TALL-104 has been shown to display potent antitumor effects in several animal models with spontaneous and induced malignancies. In view of its potential future use in cancer therapy, we investigated the tolerability and target-organ toxicity of these cells in various animal species. The acute toxicity of TALL-104 cell administrations was evaluated in: (a) healthy immunocompetent mice and immunodeficient (SCID) mice bearing human tumors using multiple (up to 15) intraperitoneal (i.p.) injections, and (b) healthy dogs, tumor-bearing dogs, and healthy monkeys using multiple (up to 17) intravenous (i.v.) injections. TALL-104 cells were γ-irradiated (40 Gy) prior to administration to mice and dogs, but administered without irradiation in monkeys. Cell doses ranged from 5×10^7/kg to 10^{10}/kg for each injection. All regimens were well tolerated, the main clinical signs observed being transient gastrointestinal effects. Moderate and transient increases in liver transaminase levels were observed in all animal species. Discrete and transient leukocytosis with neutrophilia was also noted in dogs and monkeys after i.v injections of TALL-104 cells. Histological analysis revealed foci of hepatic necrosis with lympho-/mono-/granulocytic infiltration in immunocompetent mice injected i.p. with $5\times10^9-10^{10}$ cells/kg. In the same mice, the colon showed an increased number of muciparous cells and alterations in the villi structure: these alterations were completely reversed by 72 h after the last injection, while liver alterations reversed more slowly (1 week). No delayed or chronic toxicity was observed in any of the animals even when non-irradiated TALL-104 cells were administered: both immunocompetent mice and healthy dogs were found to be grossly and histopathologically normal when sacrificed (1 year and 1 month after the last TALL-104 injection respectively). TALL-104 cells did not persist in these hosts. In addition, monkeys showed no molecular signs of TALL-104-cell-induced leukemia in their blood 1 year after the last cell injection. Despite immunosuppression, most of the tumor-bearing dogs as well as the healthy dogs and monkeys developed both humoral and cellular immune responses against TALL-104 cells. The data derived from these preclinical studies suggest that administration of high doses of irradiated TALL-104 cells is well tolerated and would be unlikely to induce severe toxicity if applied in clinical trials to the treatment of patients with refractory cancer.

Key words Cell therapy · MHC-non-restricted cytotoxic T cell line · Acute toxicity · Chronic toxicity · Biodistribution · Immunological effects · Hematological effects

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Introduction

Clinical trials have demonstrated the antitumor efficacy of lymphokine-activated killer (LAK) cells in conjunction with recombinant human interleukin-2 (rhIL-2) in patients with renal cell cancer and melanoma [1–3]. However, this therapy has also produced significant toxic side-effects, largely ascribed to the administration of IL-2, which can lead to capillary leak syndrome and, in turn, life-threatening anasarca and multi-organ system dysfunction [1–6]. Although completely reversible upon termination of IL-2 therapy, the increased vascular permeability has led to varying degrees of interstitial pulmonary edema during treatment, producing major respiratory compromise in

some cases [7]. Unfortunately, the antitumor effects of IL-2 and its toxicity are both dose-related; this constitutes an important drawback to its clinical use. Numerous phase I and II clinical trials have been conducted to evaluate different routes of administration [8] and dose schedules [9, 10] for IL-2, and combinations of IL-2 with other cytokines [11–14], drugs [15–18], or cells (LAK, tumor-infiltrating lymphocytes) [19–21] in efforts to minimize the side-effects of IL-2 administration without compromising its potency as an antitumor agent. Thus far, these studies have yielded promising but inconclusive results.

We have developed a new cell-therapy approach to cancer that might overcome the limitations of LAK/IL-2 therapy because it does not require the concomitant administration of exogenous cytokines such as IL-2 for efficacy. This approach is based on the use of the IL-2-dependent human leukemic T cell line TALL-104 (CD3/TCR$\alpha\beta^+$, $CD8^+$, $CD16^-$), which was established and characterized in our laboratory [22–25]. These cells are endowed with MHC-non-restricted killer activity against a broad range of tumors across several species, while sparing cells from normal tissues [22–25]. Tumor cell lysis by TALL-104 cells occurs by a perforin-mediated pathway or can be Fas-dependent. Moreover, cytokines released by TALL-104 cells upon contact with tumor targets [such as interferon γ (IFNγ), tumor necrosis factor (TNF)α, TNFβ and transforming growth factor (TGF)β] exert cytostatic effects on tumor cell growth. We have previously shown that γ irradiation (40 Gy) does not significantly affect TALL-104 cell cytotoxic activity and cytokine secretion [26]. Irradiated TALL-104 cells are very effective in purging bone marrows from leukemic cells both in vitro and in immunocompetent mouse models [27, 28]. When used in adoptive-transfer experiments, γ-irradiated (40 Gy) TALL-10^4 cells induced regression of transplantable hematopoietic and nonhematopoietic tumors in mouse models and of spontaneous cancers in dogs [29–31].

In the present study, we investigated the tolerability and potential target organ toxicities of irradiated and non-irradiated TALL-104 cells administered i.p. and i.v. into animals of different species.

Materials and methods

Cell lines

Human tumor cell lines (erythroleukemia K562, glioblastoma U87-MG, lung carcinoma A549, melanoma WM451, prostatic carcinoma DU-145) were purchased from American Type Culture Collection (Rockville, Md.) and maintained in Iscove's modified Dulbecco's medium (IMDM; Gibco BRL, Grand Island, N.Y.) supplemented with 10% heat-inactivated fetal bovine serum (Atlanta Biologicals, Norcross, Ga.; complete medium). The TALL-104 cell line was also maintained in complete medium supplemented with 100 U/ml rhIL-2 (Chiron, Emeryville, Calif.) in a humidified incubator with 10% CO_2. All cell lines were repeatedly monitored for *Mycoplasma* contamination.

Table 1 Schedule of γ-irradiated TALL-104 cell administration in tumor-bearing SCID mice

Dose of γ-irradiated TALL-104 cells (kg^{-1})	Number of mice	Schedule of i.p. administration
10^9	20	Daily for 15 days
5×10^9	80	Daily for 10 days
10^9	80	Alternate days (total of 6×); weekly (total of 6×)

OKT3 preparation

OKT3 hybridoma cells (American Type Culture Collection) were expanded at 37 °C in 5% CO_2 in complete medium. Cells were injected (10^7 i.p.) into 6-week-old BALB/c mice (Taconic, Germantown, N.Y.) pretreated 7 days earlier with Pristane (Sigma; 0.5 ml i.p.) and sublethally irradiated (40 Gy) just prior to the hybridoma cell injection. After 10–12 days, ascites fluids were collected, pooled and purified using an IgG affinity chromatography column (mAb-Trap GII; Pharmacia, Uppsala, Sweden). The purified mAb was checked for reactivity against TALL-104 cells by immunofluorescence analysis, filtered on 0.2-μm-pore-size filters (Corning, New York, N.Y.) and stored in aliquots at −20 °C.

Mice

Six-week-old Balb/c mice (Charles River Laboratories, Wilmington, Mass.) were housed in The Wistar Institute Animal Facility in micro-insulator cages. Mice were injected i.p. with γ-irradiated TALL-104 cells (40 Gy) at a dose of 5×10^9/kg twice a day (at 8-h intervals) or at the single dose of 10^{10}/kg (10 mice per group). Mice were checked daily for clinical signs of toxicity. Blood was drawn by retro-orbital puncture with heparinized capillary tubes (Fisher Scientific, Pittsburgh, Pa.) 24 h and 1 week after the last cell injection. Complete serum chemistry screenings (including alanine aminotransferase, aspartate aminotransferase, albumin, blood urea nitrogen, creatinine, glucose, total bilirubin, Na, K, Cl) were performed. Some sera were also tested for the presence of human cytokines (see below). Two mice per group were sacrificed at 24 h and 72 h and organs were removed for histopathological analysis. The remaining mice were maintained for analysis of long-term toxicity.

Five- to 6-week-old CB-17/SCID mice (Charles River Laboratories), housed in a pathogen-free environment in The Wistar Institute Animal Facility, were engrafted s.c. with the human tumor cell lines listed above. At different times after tumor cell transfer (from day 1 to day 14), mice were injected i.p. with γ-irradiated TALL-104 cells

Table 2 Laboratory tests performed on dog and monkey blood samples before, during, and after TALL-104 cell injection

Whole blood	Sera
Red blood cells	Glucose
White blood cells	Blood urea nitrogen
Differential	Creatinine
Hematocrit	Phosphorus
Hb	Calcium
Mean corpuscolar volume	Potassium
Mean corpuscolar Hb concentration	Chloride
Mean corpuscolar Hb	Carbon dioxide
Platelets	Total protein
	Albumin
	Alanine aminotransferase
	Alkaline phosphatase
	Total bilirubin
	Cholesterol
	Anion gap

Table 3 Toxicity studies in non-human primates; schedules of administration of TALL-104 cells. *M, W, F* Monday, Wednesday, Friday

Schedule			Monkey no.	Immunosuppression daily		Dose of TALL-104 cells (kg^{-1})	
				Cyclosporin A (5 mg/kg twice daily)	Methylprednisolone (0.5 mg/kg twice daily)	Non-irradiated	Irradiated
I	week 1	M	1	+	–	10^8	
	week 2	M				10^8	
	week 17						10^7 (boost)
II	week 1	W, F, M	2	+	–	$2.5-5\times10^8$	
			3	+	–	$2.8-5\times10^8$ + OKT3 (0.1 μg/10^6 cells)	
	week 10						10^7 (boost)
III	week 1	M→F (daily)	4	+	+	$5\times10^7-5\times10^8$	
	week 4						10^7 (boost)
	week 1		5	+	+		
	week 4						10^7 (boost)

following the doses and schedule of administration detailed in Table 1. Blood was collected at various intervals (24 h after a single injection and 1–2 weeks after the last injection of a cycle). Sera were separated by centrifugation and the presence of human cytokines was tested in the pooled sera (see below). Some mice were sacrificed at different times after the last TALL-104 cell injection (see Results) and subjected to necropsy. Some mice that appeared cured from their tumors upon TALL-104 cell therapy were maintained for 1 year and thereafter sacrificed for histopathological evaluation of long-term toxicity (see below).

Biodistribution of TALL-104 cells in Balb/c mice

TALL-104 cells (2.5×10^7/mouse) were labeled overnight in a 37 °C humidified 10% CO_2 incubator with 0.25 mCi $Na_2[^{51}Cr]O_4$ (DuPont NEN, Boston, Mass.). After three washes in IMDM, cells were γ-irradiated and resuspended in 500 μl phosphate-buffered saline (PBS); a 50-μl aliquot was assessed for isotope incorporation in a γ counter and the total injected radioactivity was calculated. Balb/c mice were injected i.p. (n = 10) and i.v. (n = 10) with labeled cells and sacrificed at different times after injection (2 mice per assay at 2, 8, 24, 48, and 72 h). All major organs were collected, weighed, and assessed for radioactivity in a γ counter. Results are expressed as cpm/g organ collected.

Healthy dogs

Three dogs, 4-month-old siblings bred at the School of Veterinary Medicine of the University of Pennsylvania (Philadelphia, Pa.), were used in this study. Two dogs were male, one was female and all weighed 9–11 kg. All three dogs received two doses of cyclosporin A (CsA, each dose 15 mg/kg, per os) as immunosuppressive agent to prevent rejection of the xenogeneic cells. One CsA dose was given 24 h before TALL-104 cell injection and one just before cell administration. The dogs were sedated with oxymorphone and injected i.v. with γ-irradiated TALL-104 cells (10^8/kg in 50 ml saline) by slow (30 min) infusion through a venous catheter positioned in a distal vein of the front leg. The dogs were carefully observed for signs of acute toxicity during the infusion and in the following month. Blood samples were taken at 0, 4, 8, 24, and 48 h after TALL-104 cell injection. Blood cell counts and a complete serum chemistry profile were performed (Table 2). All laboratory tests were repeated weekly for 1 month. At that time, dogs were euthanized with a lethal dose of pentobarbital (150 mg/kg) and a complete necropsy was performed.

Tumor-bearing dogs

Thirty dogs bearing spontaneous malignancies of various histological types (including lymphoma, breast cancer, malignant histiocytosis, nasopharyngeal squamous cell carcinoma, melanoma, mast cell tumor, hemangiosarcoma) were entered in the study.

Nineteen dogs had advanced refractory disease and 11 dogs were in clinical remission at the time of enrollment in the TALL-104 study. All treatments were discontinued at least 1 week before cell administration. Dogs were divided into three groups according to the injection schedule. In schedule I, γ-irradiated TALL-104 cells were administered every other day at a constant dose of 10^8/kg for 2 consecutive weeks followed by four weekly boosts. CsA was administered at a dose of 10 mg/kg p.o. daily, starting from the day before TALL-104 cell administration throughout the first 2 weeks of injections. In the following 4 weeks, CsA was given to the dogs only the day before and on the same day as the TALL-104 injection. In schedule II, CsA (5 mg/kg) was administered twice a day and irradiated TALL-104 cells (10^8/kg i.v.) were given daily for 5 consecutive days. In schedule III, CsA was withheld and irradiated TALL-104 cells (10^8/kg i.v.) were administered daily for 5 consecutive days followed by single monthly injections at the same dose. The number of cell infusions given to each dog varied from 4 to 17 and the total number of cells infused in each dog ranged from 4×10^9 to 10^{11}. Some of the dogs were hospitalized during the cell treatment but most were treated as outpatients. Clinical signs of acute toxicity (such as fever, chills, hypotension, diarrhea, vomiting, etc.) were monitored during and after each cell injection. For the dogs treated as outpatients the owners were properly instructed to report on the well-being of their pets during cell treatment. Complete cell counts and serum chemistries (Table 2) were performed on samples obtained from the dogs before the study and before each cell administration.

Non-human primates

Five young adult male cynomologus monkeys (3–6 years old) weighing between 3 kg and 6.3 kg were housed individually in a room air-conditioned to 25 ± 2 °C with a relative humidity of 50% ± 20% at the Memorial Sloan-Kettering Cancer Center (New York, N.Y.). The animals were maintained on a 12-h light/dark cycle and were provided with commercial primate chow and water ad libitum; fruit was given daily. All of the animal experiments were conducted in compliance with GLP regulations for nonclinical laboratory studies issued by the United States FDA, with the Animal Welfare Act, and with the guide for the Care and Use of Laboratory Animals, prepared by the Institute of Laboratory Animal Resources, National Research Council. Prior to drug/cell infusion, the monkeys were surgically implanted with a Fogarty catheter through the jugular vein, to reach the right atrium,

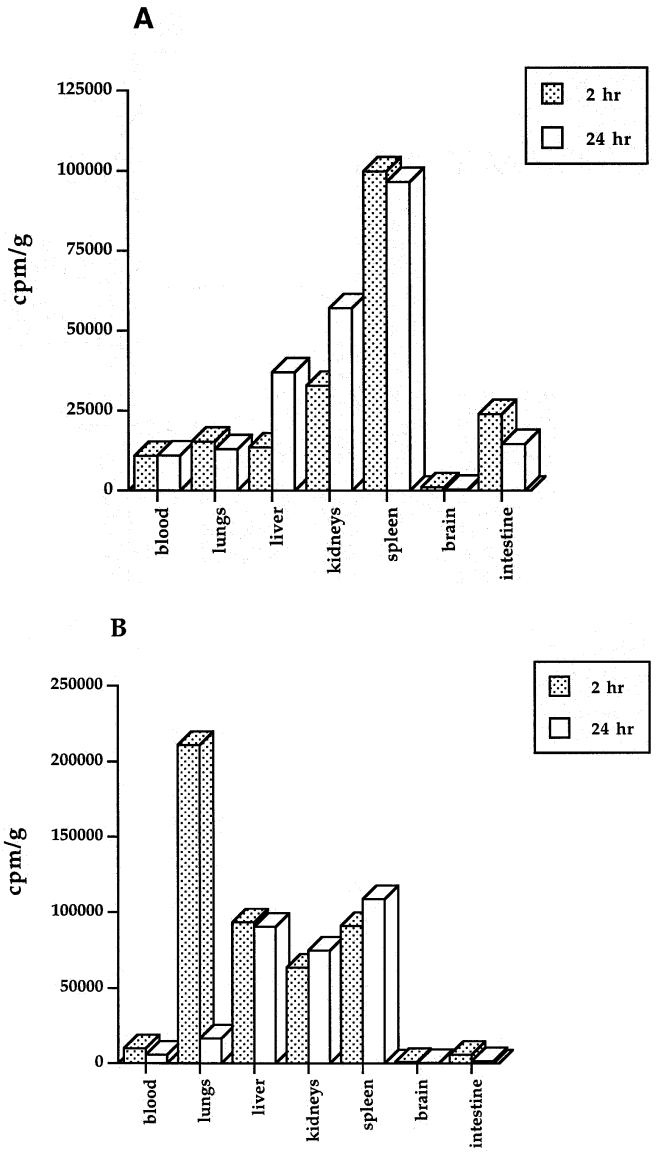


Fig. 1 Biodistribution of ^{51}Cr-labeled TALL-104 cells in mouse tissue. Balb/c mice were injected i.p. (**A**) or i.v. (**B**) with 2×10^7 ^{51}Cr-labeled TALL-104 cells. Mice were sacrified 2 and 24 h after injections, and lungs, livers, spleens, kidneys, brains, blood, and intestines removed, weighed, and assessed for radioactivity using a gamma scintillation counter

facilitating the repeated CsA and TALL-104 cell injections and blood sampling required by the experimental design. The doses of irradiated or unirradiated TALL-104 cells given and the schedules of cell injections and immunosuppressive therapy are detailed in Table 3. Clinical observations were recorded daily (before, during, and after each injection) and the animals were weighed weekly. Blood samples were withdrawn at different intervals for assessment of complete blood cell counts and serum chemistry profiles. Sera were also analyzed for the presence of human cytokines (see below).

Histopathology

Organs removed from mice (Balb/c and SCID) and healthy dogs at necropsy (including lungs, liver, spleen, kidneys, intestine, ovaries or testis, brain, lymph nodes, and spinal cord) were fixed in 10% buffered formalin (Fisher), paraffin-embedded, sectioned, and stained with hematoxylin/eosin for histopathological analysis.

Cytokine assays

Levels of human IFNγ, TNFα, TNFβ and granulocyte/macrophage-colony-stimulating factor (GM-CSF) were measured in serum samples obtained at different times before and after TALL-104 cell administrations (see above) using cytokine-specific enzyme-linked immunosorbent assay (ELISA) kits (Endogen, Boston, Mass.), according to the manufacturer's procedure. The sensitivity of the assay was 20 pg/ml for IFNγ and TNFα, 8 pg/ml for TNFβ and 7.8 pg/ml for GM-CSF.

Immunological monitoring in dogs and monkeys

Serum and peripheral blood mononuclear cell (PBMC) samples [separated from whole blood by Accu-Prep (Accurate Chemical, Wesbury, N.Y.) lymphocyte gradient centrifugation] were obtained at different times before and after TALL-104 cell injections, and monitored for the development of humoral and cellular immune responses, respectively, against TALL-104 cells. Sera were diluted at 10^{-3} in fluorescence-activated cell sorting (FACS) buffer (Ca^{2+}- and Mg^{2+}-free PBS with 0.1% NaN_3 and 2% IgG-free horse serum) and incubated with TALL-104 cells (10^5/well) in a 96-well round-bottomed plate for 1 h at room temperature. After three washings in FACS buffer, a fluorescein-isothiocyanate-conjugated rabbit anti-dog IgG or anti-monkey IgG (whole molecule; Sigma) was added at 2×10^{-2} for 1 h at 4 °C. At the end of the incubation, cells were washed, resuspended in 150 µl FACS buffer, and analyzed by flow cytometry using an Ortho cytofluorograph cell sorter. The development of a TALL-104-specific cellular immune response was monitored in dogs and monkeys by testing the cytotoxic activity of the dogs' PBMC against ^{51}Cr-labeled TALL-104 cells in an 18-h ^{51}Cr-release assay [23]. MHC-non-restricted killing activity was measured in the same assays by testing dogs' PBMC against K562 cells.

PCR analysis

The presence and persistence of circulating TALL-104 cells in dogs and monkeys were evaluated by polymerase chain reaction (PCR) analysis of frozen aliquots of DNA extracted as described [32] from PBMC obtained at various intervals before and after TALL-104 cell injections. Two primers specific for the human minisatellite region YNZ.22 [32] were used. 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 [32].

Results

Migration of i.v/i.p. injected TALL-104 cells in murine tissues

Radiolabeled, irradiated TALL-104 cells, injected i.p. into Balb/c mice, were detected mainly in the spleen within 2 h after injection (Fig. 1A) and accumulated also in the liver and kidneys 24 h later. The distribution of TALL-104-cell-associated radioactivity remained unchanged from 24 h to 72 h after transfer (not shown). Upon i.v. injection, most of the radioactivity at 2 h was localized in the lungs (Fig. 1B) whereas, after 24 h, TALL-104 cells were detected primar-

Table 4 Laboratory screens of healthy dogs injected i.v. with γ-irradiated TALL-104 cells (10^8/kg). *WBC* white blood cells, *PMN* polymorphonuclear leukocytes, ND not dance

Dog no.	Cells	Cell count (CC)		
		0 h	8 h	24 h
1	WBC	11100	17170	10400
	PMN	4551	10620	5720
	Lymphocytes	6105	6549	4160
	Monocytes	111	531	312
	Eosinophils	333	ND	208
2	WBC	12600	14700	12000
	PMN	7560	8820	6960
	Lymphocytes	4534	5733	4680
	Monocytes	504	147	240
	Eosinophils	ND	ND	120
3	WBC	11700	16400	10000
	PMN	7020	10660	5900
	Lymphocytes	4212	4100	3700
	Monocytes	117	820	200
	Eosinophils	351	192	200

ily in the liver, kidneys, and spleen, with much lower levels of radioactivity recovered from the lungs. Although the total recovery of radiolabeled TALL-104 cells was low after 48 h and 72 h (20%–40%), most of the detectable radioactivity was still in the liver and spleen (not shown). The same migration pattern was seen with non-irradiated TALL-104 cells (not shown) thus proving that irradiation does not affect motility or diapedesis of these cells.

Clinical observations in mice post-adoptive transfer

No clinical signs of acute toxicity were detected in any of the 180 SCID mice bearing human tumors (see Materials and methods) that were injected i.p. with different doses and schedules of administration of γ-irradiated TALL-104 cells (Table 1). Out of 80 tumor-bearing SCID mice successfully treated with 10^9/kg irradiated TALL-104 cells injected i.p. on alternate days for a total of 6 injections and weekly thereafter for a total of 6 weeks (Table 1), 25 were maintained for 1 year. These mice never developed any kind of delayed or chronic toxicity, including leukemia.

Clinical acute toxicity was noted in a group of 10 healthy Balb/c mice injected i.p. twice daily with irradiated TALL-104 cells at a dose of 5×10^9/kg and in a second group of 10 Balb/c mice treated with a single i.p. injection of 10^{10} cells/kg. Lethargy, ruffled fur, hunched posture and severe diarrhea with significant weight loss (from 23.1 ± 0.4 g to 17.8 ± 0.9 g mean weight) were observed within 24 h of the last TALL-104 injection in all mice. However, all symptoms, with the exception of weight loss, were transient and regressed completely in 48 h in 95% of the mice. One mouse died as a result of acute toxicity within 24 h of the cell infusion in the second group of mice treated with the highest dose. One week after the end of the injections the mean weight was 22.8 ± 0.4 g.

Table 5 Examples of leukocytosis with neutrophilia in tumor-bearing dogs before, during, and after TALL-104 therapy

Dog no.	White blood cells (l^{-1})	Granulocytes (%)
1	Before = 6.7×10^9	88
	During = 21.2×10^9	91 (highest value)
	After = 7.1×10^9	85
2	Before = 10.9×10^9	83
	During = $43.9.1\times10^9$	98 (highest value)
	After = 7.8×10^9	81
3	Before = 4.5×10^9	56
	During = 15.2×10^9	94 (highest value)
	After = 6.7×10^9	57
4	Before = 10.2×10^9	70
	During = 18×10^9	83 (highest value)
	After = 7.1×10^9	67

Clinical observations in dogs and primates

No acute toxicity was observed in the three healthy dogs after a single i.v. injection of irradiated TALL-104 cells (10^8/kg). In the 30 tumor-bearing dogs, no life-threatening acute reactions were observed during or after TALL-104 cell injections, except for an isolated reaction seen in a female dog with lung metastatic breast cancer upon the second TALL-104 cell injection. The dog collapsed 10 min after the start of the infusion, showing a weak pulse and decrease in capillary refill time. The infusion was immediately stopped, and the dog was treated with dexamethasone i.v. Once the dog was stabilized, the cell infusion was completed at a slower rate. After this episode, the dog was premedicated with dexamethasone before each TALL-104 cell injection with no further adverse reactions. Mild vomiting, responsive to antiemetics, and diarrhea were seen in one-third of the dogs.

No clinical signs of acute toxicity were detected in the four monkeys injected with non-irradiated TALL-104 cells during and after cell administration. Co-administration of OKT3 mAb (known to be a potent stimulus for lymphokine-release by TALL-104 cells) did not add any toxicity. The fifth monkey did not receive TALL-104 cells and was used as a control for immunosuppression-related toxicities (Table 3).

Hematological effects of adoptive transfer of TALL-104 cells

Transient (and in one case dramatic) alterations were seen in the results of the hematological tests of all 3 healthy dogs 8 h after the single TALL-104 cell infusion, consisting of an increase in white blood cell counts associated with an increase in the absolute number of granulocytes (Table 4). These alterations normalized within 24 h after infusion. Discrete leukocytosis with neutrophilia was also noted in about half of the tumor-bearing dogs: the number of white blood cells increased rapidly after the infusion, reaching 1.7–4 times the baseline levels with 83%–98% neutrophils

Table 6 Laboratory tests performed on blood samples of monkeys injected with TALL-104 cells. See Table 3 for schedule of cell administration for each monkey. *BUN* blood urea nitrogen, *ALT* alanine aminotransferase, *AST* aspartate aminotransferase, *WBC* white blood cells, *PMN* polymorphonuclear leukocytes, *Lym* lymphocytes

Monkey no.	Test	Day 1	Day 4	Day 7	Day 14
1	BUN mg/dl	20	16	20	–
	ALT IU/l	153	137	128	–
	AST IU/l	134	92	78	–
	WBC$\times 10^3$/cc	7.3	6.3	8.9	–
	PMN (%)	81	49	63	–
	Lym (%)	18	32	28	–
2	BUN mg/dl	10	29	12	–
	ALT IU/l	54	65	46	–
	WBC$\times 10^3$/cc	10.4	10.4	13.7	–
	PMN (%)	18	21	12	–
	Lym (%)	76	77	86	–
3	BUN mg/dl	10	21	12	–
	ALT IU/l	39	47	41	–
	WBC$\times 10^3$/cc	10.4	18.1	9.9	–
	PMN (%)	64	50	49	–
	Lym (%)	34	48	51	–
4	BUN mg/dl	21	18	14	–
	ALT IU/l	56	76	107	54
	AST IU/l	23	83	55	20
	WBC$\times 10^3$/cc	12.6	25.8	19.6	13.1
	PMN (%)	57	58	58	54
	Lym (%)	45	36	43	43
5	BUN mg/dl	20	18	19	17
	ALT IU/l	58	58	58	56
	WBC$\times 10^3$/cc	10.9	14.1	11.7	11.1
	PMN (%)	54	68	63	60
	Lym (%)	42	32	36	39

at 24 h, and returned to normal within 24–48 h after the last cell infusion (see examples in Table 5). No significant correlation was found between neutrophilia and schedule administration.

No significant hematological effects were observed on monkey no. 1, injected with 10^8/kg non-irradiated TALL-104 cells once a week for a total of two infusions, together with CsA (5 mg/kg twice daily), or in monkey no. 5 (control receiving only immunosuppressive drugs). However, the absolute number of white blood cells increased from 4 days after infusion in monkey no. 3 (who was injected with 2.5×10^8–5×10^8/kg non-irradiated TALL-104 cells on alternate days for a total of three injections together with CsA, (5 mg/kg twice daily), and in monkey no. 4 (who was injected with escalating doses of TALL-104 cells, ranging from 5×10^7–10^8/kg, daily for 7 days, together with 5 mg/kg CsA and methylprednisolone, 0.5 mg/kg twice daily). By day 7, this leukocytosis had resolved in monkey no. 3 but remained elevated in monkey no. 4 because of the different schedule of cell administration. The test normalized in this monkey 1 week after the last injection (Table 6).

Effects on serum parameters

Analysis of sera collected from Balb/c mice 24 h after the last injection showed increased liver transaminases, decreased total bilirubin and albumin, and hypokalemia and hyperchloremia (Table 7). These values normalized by day 7.

Chemistry profiles assayed on sera taken from healthy dogs 0, 4, 8, 24, and 48 h after the single TALL-104 cell injection and then weekly for 1 month revealed no abnormalities (not shown).

An increase in liver transaminases (highest alanine aminotransferase values, 439 IU; normal dog range = 0–77

Table 7 Serum chemistries on healthy immunocompetent Balb/c mice injected i.p. with TALL-104 cells (10^{10}/kg). All chemistry tests were run on pooled sera from 5 mice. *ALB* albumin, *TBil* total bilirubin

Test	0 h	24 h	7 days	Normal mouse range
ALT (IU/l)	46	180	39	28–132
ALB (g/dl)	3.74	2.41	3.61	2.5–4.8
TBil (mg/dl)	0.75	0.00	0.6	0.1–0.9
K^+ (mmol/l)	5.30	4.52	5.1	4.7–6.4
Cl^- (mmol/l)	111.6	128.7	121.1	92–120

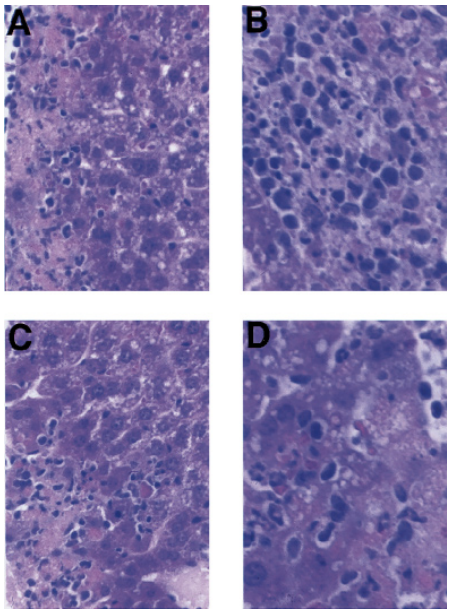


Fig. 2 A–D Histopathological analysis of livers from two representative Balb/c mice injected i.p. with 10^{10}/kg irradiated TALL-104 cells. Necrotic foci with lymphocytic/monocytic/neutrophilic infiltration are evident beneath the liver capsule. **A, B** Alterations at 24 h; **C, D** liver changes at 72 h. Hematoxylin/eosin staining; magnification 200× (**A, C**) and 400× (**B, D)**

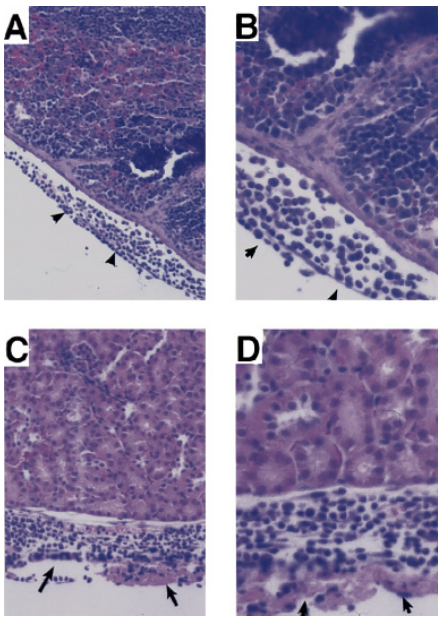


Fig. 3A–D Histopathological analysis of spleen (**A, B**) and kidney (**C**, **D**) from a representative Balb/c mouse injected i.p. with 10^{10}/kg irradiated TALL-104 cells 24 h before sacrifice. Note the presence of a layer of TALL-104 cells between the visceral peritoneum and the splenic capsule (*arrows*; **A, B**) or the renal capsule (*arrows*; **C, D**) but no infiltration into the organs. Magnification 200× (**A, C**) and 400× (**B, D**)

IU) was seen in fewer than 10% of the tumor-bearing dogs in the study, sometimes associated with a transient increase

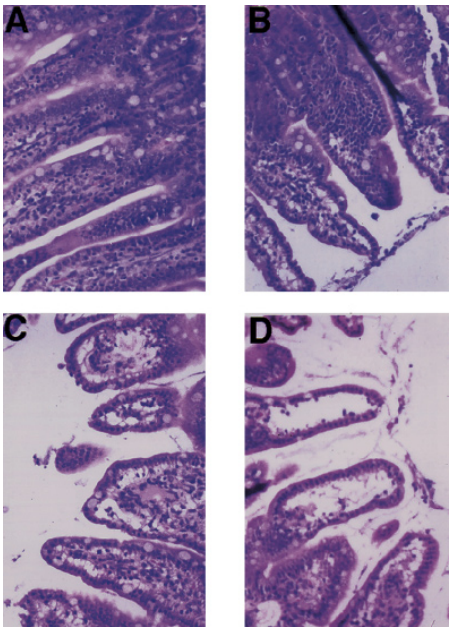


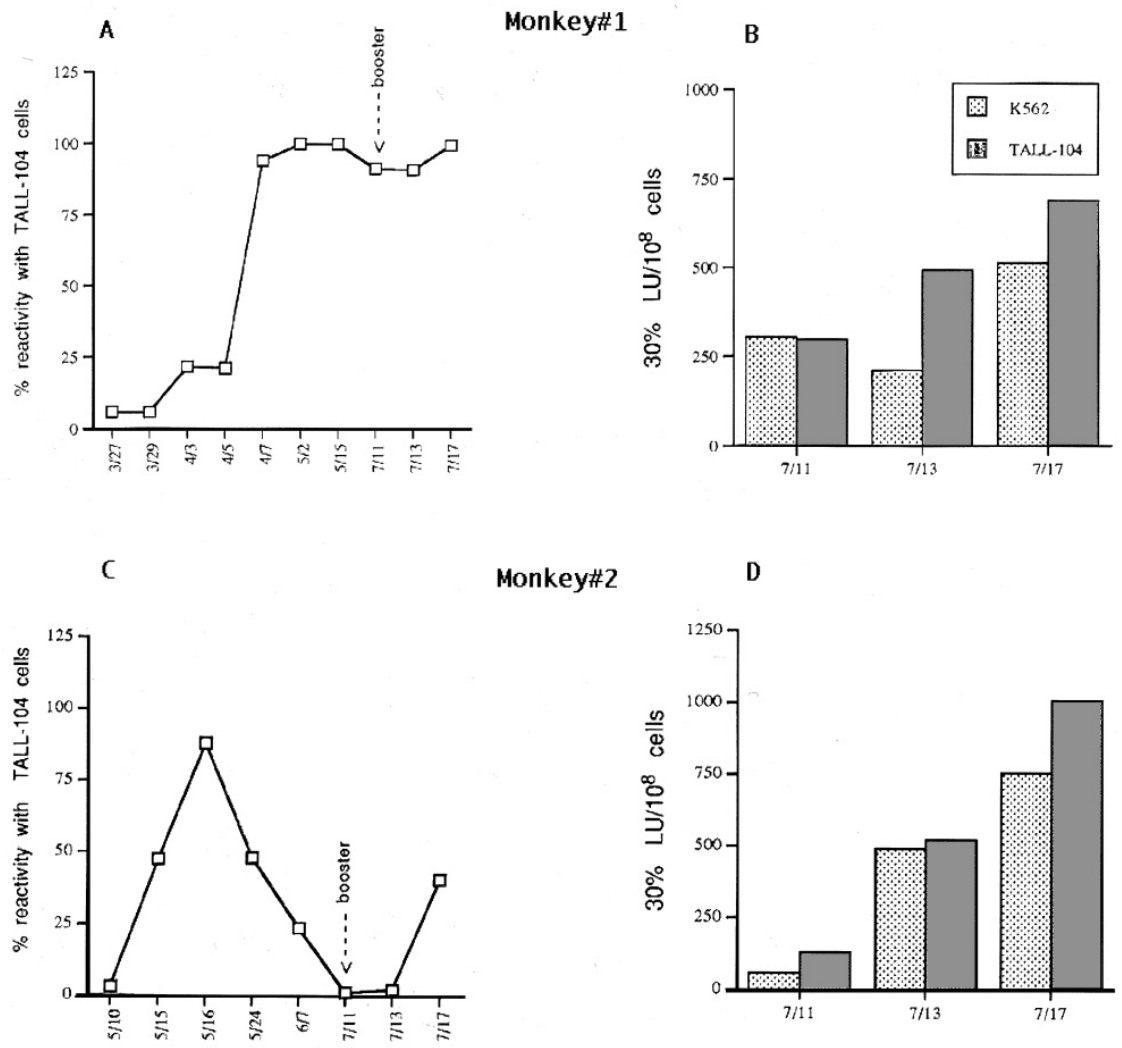
Fig. 4A–D Histopathological analysis of different segments of the intestine from a representative Balb/c mouse that developed diarrhea after i.p. injection of irradiated TALL-104 cells (10^{10}/kg). **A** Duodenum: despite the general increase in the number of muciparous cells in the villous epithelium, the structure of the villi is otherwise well preserved. **B** Illeum: modest alterations in some of the villi, limited to the luminal part. **C** Colon transversum: the alterations in the villi are more diffuse and severe. **D** Colon distale: dramatic destruction of most of the villous structure. All of these anatomopathological alterations were completely reversible within 72 h after TALL-104 cell injection. Hematoxylin/eosin staining; magnification 200×

in alkaline phosphatase (highest value, 1116 IU; normal dog range = 0–400 IU) and in total bilirubin (highest value, 4.41 mg/dl; normal dog range = 0–0.50 mg/dl). These alterations were transient and returned to normal levels as soon as cell injections were halted.

Small and transient increments in liver transaminases and blood urea nitrogen were noted in the sera of monkeys 2, 3 and 4, which nevertheless remained within the normal range (Table 6). These tests were back to normal by day 7 in monkey no. 2 and by 1 week after the last injection in monkey no. 4, because of the different schedule of cell administrations.

Macroscopic observations

Necropsy was performed on some of the SCID and Balb/c mice and in the 3 healthy dogs. No macroscopic findings appeared related to TALL-104 cell administration except for splenomegaly, which was noted in tumor-bearing SCID mice injected i.p. with 10^9/kg irradiated TALL-104 cells daily for 15 consecutive days or with 5×10^9/kg for 10 consecutive days. Notably, mice sacrificed within 2 weeks of the last cell injection had a significantly enlarged spleen (the mean spleen weight of untreated mice was 0.028 ± 0.005 g, whereas the mean spleen weight of treated mice was 0.102 ± 0.010 g) due, possibly, to the effects of



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cytokines (such as GM-CSF) produced by TALL-104 cells during tumor interaction and cross-reacting with lympho-hematopoietic murine cells. Both histology and PCR analyses excluded the direct presence of TALL-104 cells in these spleens at that time (not shown).

Microscopic observations

The splenomegaly noted on macroscopic evaluation of tumor-bearing SCID mice receiving multiple injections of irradiated TALL-104 cells was found to be associated with an increase of granulocytopoiesis in the spleen (not shown). Otherwise, these mice showed no histopathological abnormalities other than those attributed to the implanted tumors. By contrast, Balb/c mice sacrificed 24 h and 72 h after the last i.p. injection had histological evidence of foci of hepatic necrosis with lymphocytic, monocytic, and granulocytic infiltration beneath the liver capsule and inside the parenchyma (Fig. 2). Subcapsular infiltrates of lymphoid cells (likely TALL-104 cells) were also detected in the spleens and kidneys (Fig. 3A, B and C, D respectively), but were never associated with necrosis and/or invasion of the parenchyma. The duodenum, ileum and rectum showed no significant histological abnormalities; however, dramatic histological changes were seen in the colon, consisting of an increased number of muciparous cells together with alterations in the structure of the villi (Fig. 4). The intestinal abnormalities were reversible (not detectable 72 h after the last cell injection), while the liver alterations were slower to repair, still being visible at 72 h (Fig. 2C, D). These observations were consistent with laboratory findings of persistent elevated transaminases at 48 h and 72 h, which slowly normalized over 1 week (see above).

Histopathological analysis of tissues obtained from the 3 healthy dogs sacrificed 1 month after a single TALL-104 cell injection demonstrated no organ toxicity (not shown).

Serum levels of human cytokines

No detectable levels of human (TALL-104 released) cytokines (TNFα, TNFβ, IFNγ, and GM-CSF) were found in

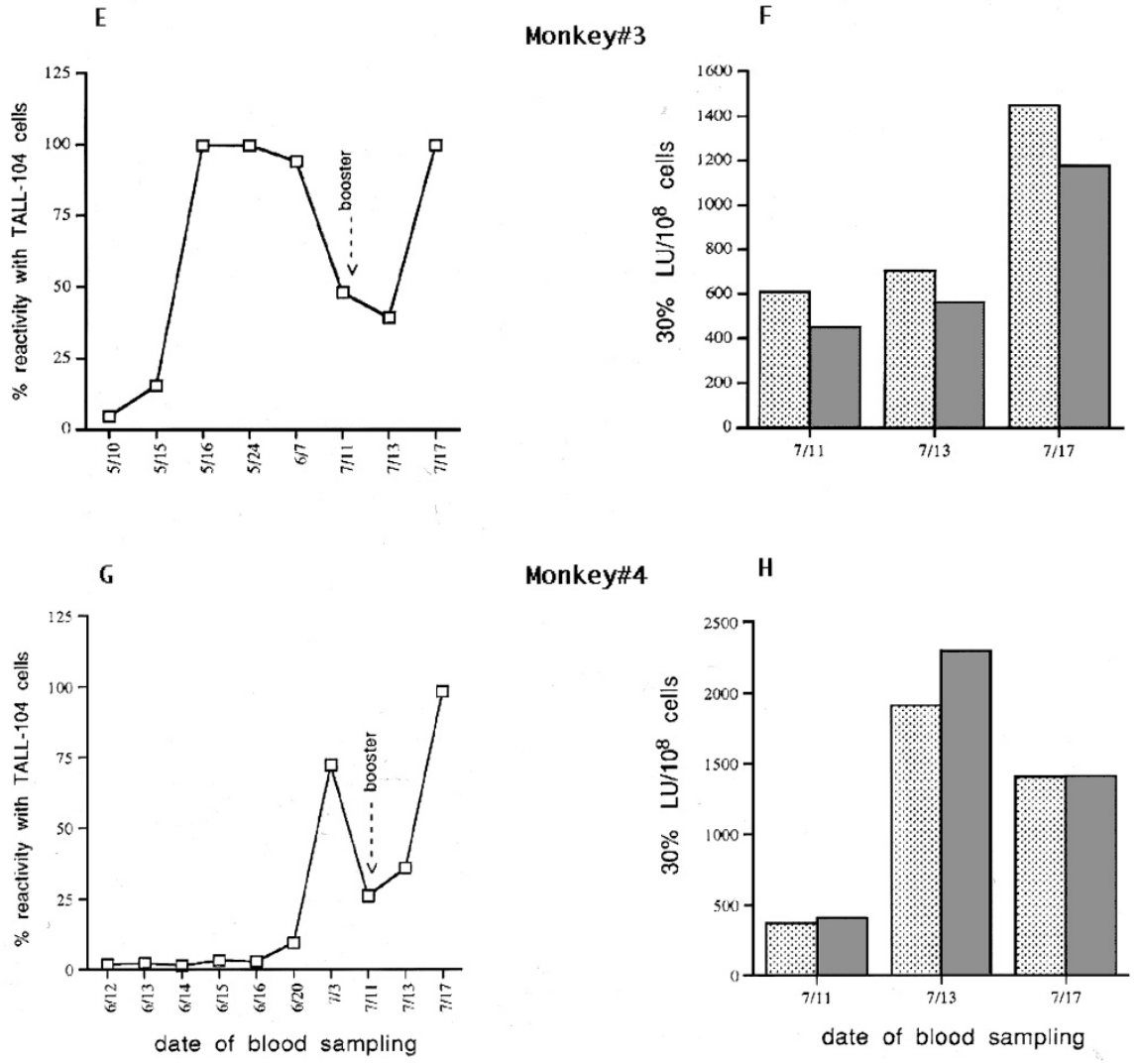

Fig. 5A–H Humoral (**A, C, E, G**) and cellular (**B, D, F, H**) immune responses against TALL-104 cells developed by 4 monkeys injected i.v. with different doses of non-irradiated TALL-104 cells (see Table 3 for dose and schedule of cell administration). Blood samples were obtained on the indicated dates. The presence of antibodies against TALL-104 cells was tested in monkey sera by fluorescence-activated cell sorting analysis as described in Materials and methods; monkey peripheral blood mononuclear cells (PBMC) were tested for cytotoxicity against K562 and TALL-104 cells in an 18-h ^{51}Cr-release assay

mouse sera (both SCID and Balb/c) collected 24 h after TALL-104 cell injection. However, significant levels of these cytokines were detected in the sera of some tumor-bearing dogs 24 h after TALL-104 cell injections (IFNγ, 30–120 pg/ml, TNFα, 25–132 pg/ml, and TFNβ, 40–150 pg/ml), but these levels were not associated with any clinical toxicity. Modest levels of TNFα and TNFβ (30–40 pg/ml) were also detected, although randomly, in monkey sera within a few hours after cell injection.

Immune response against TALL-104 cells

Despite the different immunosuppressive regimens with CsA given in association with steroids or not, virtually all healthy and tumor-bearing dogs (not shown) and monkeys (Fig. 5) developed a humoral immune response against TALL-104 cells, usually between days 8 and 12 after the first injection (Fig. 5A, C, E, G). Specific and aspecific cellular immune responses against TALL-104 and K562 cells (respectively) were demonstrated in 80% of the treated dogs (not shown) and in the monkeys (nos. 1–4) after boosting with γ-irradiated TALL-104 cells (Fig. 5B, D, F, H), but not in control monkey no. 5 (not shown).

Detection of circulating TALL-104 cells

PCR amplification of the human minisatellite region YNZ.22 was performed on PBMC isolated from the dogs and monkeys at different intervals before, during, and after cell therapy to document the time of appearance and the kinetics of disappearance of non-irradiated TALL-104 cells from the circulation. Circulating TALL-104 cells were always detectable in tumor-bearing dogs (not shown) and

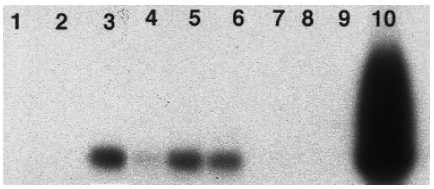

Fig. 6 Polymerase chain reaction amplification of the minisatellite region YNZ.22 performed on PBMC of monkey no. 1, obtained before and at different times after TALL-104 cell injections. *Lanes: 1* water (negative control); *2* time 0 first injection; *3* 4 days after; *4* time 0 second injection; *5* 4 h after; *6* 2 days later; *7* 4 days later; *8* 16 days later; *9* 4 months later; *10* TALL-104 cells (positive control)

in monkeys (Fig. 6) for a maximum of 1 week, irrespective of whether the TALL-104 cells were irradiated or not.

Discussion

The MHC-non-restricted cytotoxic TALL-104 cell line holds promise in future management of cancer as shown in several animal models with spontaneous and induced malignancies [29–31]. The present study was designed to investigate acute and long-term toxicities related to adoptive transfer of TALL-104 cells in different animal species using different routes and schedules of administration. The results show that, in the experimental conditions chosen, TALL-104 cell administration is a safe procedure not associated with major clinical toxicities. In fact, significant clinical toxicity was induced only in mice injected i.p. with very high doses of irradiated cells (approximately 100 times higher than the dose shown to have antitumor activity in vivo) [31]. The toxicity was limited to the gastrointestinal tract (diarrhea) and was completely reversible within 48 h of the last cell injection. Because such a large number of cells could be administered to the mice only i.p. and not systemically (without causing lethal pulmonary embolism), we cannot exclude the possibility that the gastrointestinal toxicity reflected local irritation of the intestine rather than an actual systemic toxic effect. In this regard, biodistribution studies in mice injected i.p. with radiolabeled TALL-104 cells (2.5×10^7/mouse) showed a high accumulation of radioactivity in the animals' intestines at 24 h (Fig. 2A). By contrast, gastrointestinal radioactivity was only marginal when the mice were injected i.v. with the same cell dose (Fig. 2B). Vomiting and diarrhea were also observed during i.v. TALL-104 cell administration in around 10% of tumor-bearing dogs, but these symptoms were always mild and easily controlled with appropriate therapy. The same gastrointestinal side-effects have been described in 80% of human patients during LAK/IL-2 therapy [33]. Abnormalities in liver function tests were observed in all species tested in this study, independent of the route and schedule of TALL-104 cell administration. However, in all cases, values returned to baseline 72 h to 1 week after the last cell injection, indicating that the insult to the liver was transient and reversible. Similarly, LAK/IL-2 therapy has been associated with altered hepatic functions [34], and it has been suggested that IL-2-activated lymphocytes are hepatotoxic; the strong correlation between peak lymphocyte counts after IL-2 priming and serum transaminase levels supports this contention [35]. As in the case of TALL-104 cells, the LAK-cell-induced liver abnormalities were transient and reversible.

None of the hematological abnormalities associated with IL-2/LAK therapy, including anemia, transient lymphopenia followed by rebound lymphocytosis, eosinophilia, and thrombocytopenia with coagulation disorder [36], were observed in the present study. However, various degrees of leukocytosis with relative neutrophilia were detected in both dogs and monkeys injected with TALL-104 cells. Some of the tumor-bearing dogs showed white blood cell counts two to five times above baseline levels in 24 h, with up to 99% neutrophils, perhaps reflecting the high levels of GM-CSF produced by TALL-104 cells in response to tumors [24]. However, we did not detect this cytokine in serum samples from TALL-104-treated animals at different intervals after cell administration, possibly because of (1) the time of sampling (24 h after cell injection might be too late to detect GM-CSF in the serum since secretion in vitro peaks at 8 h after the stimulus is applied); (2) the sensitivity threshold of our ELISA test (7.8 pg/ml may be insufficient); and/or (3) the fast metabolism and removal of the cytokine from the circulation. On the other hand, significant levels of IFNγ, TNFα, and TNFβ were detected in the sera of some tumor-bearing dogs and monkeys; however, no correlation was seen between these levels and clinical toxicity.

An important observation in the present study was the total absence, during TALL-104 cell injections, of clinical toxicities associated with increased capillary permeability, a finding that contrasts with preclinical and clinical experience with IL-2/LAK therapy [1–6]. The mechanisms by which IL-2/LAK cells induce vascular leak syndrome are unknown, but evidence suggests that this effect may be mediated directly or indirectly by host lymphoid elements activated by exogenous IL-2 administration [37]. Other data show that LAK cells can bind and lyse normal human vascular and corneal endothelial cells in vitro [38]. Although TALL-104 cells have not been tested for induction of vascular leak syndrome, this seems unlikely in light of the total absence of any signs associated with this syndrome in any of the animal species tested in this study. Moreover, TALL-104 cells maintain their antitumor activity in vivo without requiring exogenous IL-2 [29–31], so that administration of TALL-104 cells as single agent in prospective clinical trials should not be associated with the toxicity seen with LAK/IL-2 therapy.

The only serious acute toxicity observed in our study was an isolated reaction, easily controlled by steroids, observed in one tumor-bearing dog during her second consecutive injection with TALL-104 cells. This type of toxicity is to be expected in protocols involving injection of xenogeneic cells. The low incidence of toxic reactions in dogs and monkeys was even more surprising, considering that CsA (given to prevent TALL-104 cell rejection) did not block the development of either humoral and cellular immunity against TALL-104 cells.

Finally, the lack of circulating TALL-104 cells months after the last injections, as documented in the dogs by PCR analysis, and in mice, by histopathological analysis of tissues from animals sacrificed 1 year after the last injection with irradiated TALL-104 cells, rules out potential delayed sideieffects such as leukemia/lymphoma induced by irradiated TALL-104 cells. It is important to note that the same studies on monkeys demonstrated that even the non-irradiated TALL-104 cells are unable to proliferate, to induce sustained chimerism, or to induce leukemia in xenogeneic hosts.

Together, our data in animal models and the results from safety testing suggest that TALL-104 cells constitute a tumoricidal effector cell population that is relatively nontoxic against normal tissues in vivo. These studies, combined with previously reported preclinical studies, indicating the antitumor effects of irradiated TALL-104 cells in vitro and in vivo [29, 30], provide a basis for extending the evaluation of these cells to phase I and II trials in patients with TALL-104-sensitive tumors that are refractory to current chemo-radiotherapeutic regimens.

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