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Letter to the Editor

Pegylated TNF-α Receptor Does Not Prevent Acute Graft-versus-Host Disease in the Dog Leukocyte Antigen-Nonidentical Unrelated Canine Model

It has been suggested that cytokines in general and tumor necrosis factor α (TNF-α) in particular have important roles in the initiation of graft-versus-host disease (GVHD) [1,2]. Several recent publications have indicated that neutralization of TNF-α may be an effective treatment for acute or chronic GVHD [3-5]. Neutralization may be accomplished with a monoclonal anti-TNF-α (infliximab) or by the use of a soluble TNF receptor/immunoglobulin fusion protein (etanercept). An experimental polyethylene glycol (PEG)-treated form of the soluble TNF receptor/immunoglobulin (PEG sTNF-RI) has been recently constructed with the potential of decreasing immunogenicity and extending circulating half-life [6]. PEG sTNF-RI has been tested preclinically in the rat, cynomolgus monkey, chimpanzee, and baboon as a potential inhibitor against manifestations of rheumatoid arthritis in patients [7]. This new TNF-α inhibitor appeared to be an ideal candidate for evaluating the role of TNF-α in GVHD in the preclinical canine model.

We previously observed uniformly fatal acute GVHD in dogs when a dog leukocyte antigen (DLA) nonidentical recipient received 920-cGy total body irradiation (TBI) immediately before hematopoietic cell transplantation (HCT). On average, severe GVHD was observed within 14 days without treatment and delayed to day 20 when dogs were treated with the antimetabolite methotrexate (MTX) [8,9]. In this study, we investigated whether PEG sTNF-RI, administered in combination with MTX, could prevent or delay the initiation of GVHD in the DLA-nonidentical HCT model.

In preliminary in vitro studies, we first tested the cytotoxic effects of recombinant human TNF-α (rhTNF-α) or canine TNF-α, derived from lipopolysaccharide-treated canine peripheral blood monocytes, against the cell line WEHI using a standard tetrazolium dye (3-(4,5-dimethylthiazil-2-yl)-2,5-diphenyl tetrazolium bromide) reduction assay. Canine TNF-α and rhTNF-α were essentially equivalent in inducing cytotoxicity in a dose-dependent manner in the range of 1-8 ng/mL of rhTNF-α (Figure 1). Next, we assessed the inhibitory capacity of PEG sTNF-RI on cytotoxicity of recombinant canine TNF-α and rhTNF-α against WEHI cells. A concentration of 2.0 μg/mL of PEG sTNF-RI inhibited by 50% the cytotoxicity mediated by 2 ng/mL of canine or human TNF-α (Figure 1). Similar concentration ratios of infliximab or etanercept to TNF-α have been shown to inhibit TNF-mediated cytotoxicity against WEHI cells [10].

We then evaluated the effect of PEG sTNF-RI on 5 dogs given 920-cGy TBI followed by HCT from DLA-nonidentical donors. In the first group of 2 recipients, PEG sTNF-RI was administered twice weekly starting on the day after TBI. In the second group of 3 dogs, PEG sTNF-RI was administered every other day, starting the day before TBI. The dose of PEG sTNF-RI was 0.5 mg/kg, equivalent to the dose of etanercept recommended for children for the treatment of rheumatoid arthritis and within the range of doses of PEG sTNF-RI found to be effective in decreasing induced arthritis in Lewis rats [11]. MTX was administered at 0.4 mg/kg intravenously on days 1, 3, 6, and 11 and once weekly thereafter, as previously described [8,9]. HCT consisted of a mean of 4.6×10^8 marrow cells/kg (range, 1.8-7.8 $\times 10^8$ marrow cells/kg) infused intravenously immediately after TBI (day 0) and a mean of 2×10^8 peripheral blood buffy coat cells/kg (range, 1.25-2.7 $\times 10^8$ peripheral blood buffy coat cells/kg) infused on day 1. Toxicities were mainly gastrointestinal and hematologic owing to the effects of the 920-cGy TBI and MTX and comparable to those observed in historical controls.

The results are presented in Table 1. All dogs engrafted with complete donor chimerism. Four of 5 dogs developed acute GVHD and >30% weight loss, resulting in the need to euthanize between 13 and 18 days, independent of the PEG sTNF-RI treatment regimen. GVHD was confirmed by histopathology on necropsied tissue biopsies. The median number of days to survival was 17.5 compared with 20 days for 35 historical treated dogs in 1 study [8] and 19 days for 29 dogs in another study [9]. Statistical analysis of the data using a 2-sided Mann-Whitney U test compari-

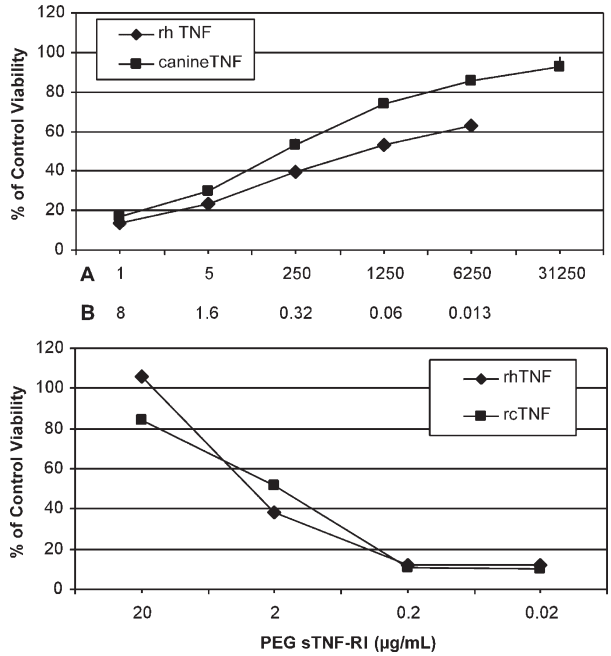

Figure 1. Cytotoxicity of rhTNF-α and canine monocyte-derived canine TNF-α against WEHI cells. Canine peripheral blood-derived monocytes were incubated for 18 hours in Iscove Modified Dulbecco Medium with 10% dog serum and 25 ng/mL lipopolysaccharide. Cytotoxicity was determined after adding monocyte-derived supernatant in 5-fold dilutions to WEHI cells (top; abscissa A units listed as reciprocal of dilution) or with 5-fold dilutions of rhTNF-α starting with 8 ng/mL (top; abscissa B units listed as nanograms per milliliter). Bottom, Titrations of PEG sTNF-RI were added to WEHI cells in the presence or absence of 2 ng/mL rhTNF-α or recombinant canine (rc) TNF-α. Toxicity for both experiments was determined by a 3-(4,5-dimethylthiazil-2-yl)-2,5-diphenyl tetrazolium bromide assay after an 18-hour incubation period.

son of nonparametric samples failed to note a significant improvement in survival in the dogs treated with PEG sTNF-RI plus MTX over dogs treated with MTX alone ($P \geq .05$). The fifth dog was euthanized on day 13, with histopathology confirming herpes virus infection.

Serum levels of PEG sTNF-RI were determined by enzyme-linked immunosorbent assay on blood samples 24 hours after treatment and daily thereafter (Figure 2). The results indicated that mean serum levels of PEG sTNF-RI, although variable for the dogs treated twice weekly and moderately less so for the dogs treated every other day, were comparable between the 2 groups. These levels of PEG sTNF-RI were similar to those reported for the same dose (0.5 mg/kg) of PEG sTNF-RI administered on a weekly basis in chimpanzees [7].

Our results indicate that PEG sTNF-RI in combination with MTX failed to induce graft-versus-host tolerance, and long-term survival was not observed in these DLA-nonidentical HCT recipients. We believe

Table 1. *Comparison of Survival in Dogs Given 920-cGy TBI, Hematopoietic Grafts from DLA-Nonidentical Unrelated Donors, and Postgrafting MTX plus PEG sTNF-RI**

Dog	Cause of Death	Grade of GVHD†			%Chimerism	GVHD Start (days)	Survival (days)
		Skin	Liver	GI Tract			
G381	GVHD	4	3,4	3,4	100	9	18
G488	GVHD	4	2	4	100	7	13
G549	GVHD	3,4	3,4	3,4	100	13	18
G574	GVHD	2,3	3	2,3	100	9	17
G581	CHV	NE‡	NE	NE	<100	0	13
						Median	17.5

Reference	Dogs, n	Treatment	Survival§	Range
8	35	MTX	20	11->102
9	29	MTX	19	9-136

TBI indicates total body irradiation; DLA, dog leukocyte antigen; MTX, methotrexate; polyethylene glycol-treated form of soluble tumor necrosis factor receptor/immunoglobulin; GVHD, graft-versus-host disease; GI, gastrointestinal; CHV, canine herpes virus; NE, no evidence by pathology.

*All dogs received 920-cGy TBI on day 0. MTX was given intravenously at a dose of 0.4 mg/kg on days 1, 3, 6, and 11. PEG sTNF-RI was given subcutaneously at a dose of 0.5 mg/kg twice weekly on days 1, 4, 8, 11, 14, 17, etc (dogs G381 and G488) or every other day starting day −1 (dogs G549, G574, and G581). Percent chimerism was determined by documentation of donor microsatellite marker polymorphisms in nucleated peripheral blood cells by polymerase chain reaction [14].

†Grade of GVHD, with 4 being the most severe, was confirmed by histopathology of autopsy tissue.

‡Dog was euthanized due to a severe case of CHV.

§Median survival in days.

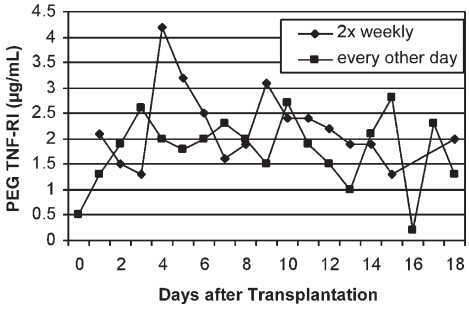

Figure 2. Mean serum concentrations of PEG sTNF-RI in dogs treated twice weekly (n = 2) or every other day (n = 3) until time of euthanasia. Concentrations of PEG sTNF-RI were determined by enzyme-linked immunosorbent assay by using the same lot of reagent as a standard in normal dog serum.

the model is valid for evaluating GVHD, because it has been used previously to identify clinically relevant drug combinations, such as MTX/cyclosporine [9], MTX/tacrolimus [12], and mycophenolate mofetil/cyclosporine [13]. A possible explanation for lack of efficacy is that the role played by TNF-α in this model of GVHD is relatively minor compared with donor anti-host major histocompatibility antigen immune recognition. In addition, earlier treatment of the recipients with PEG sTNF-RI before irradiation may be effective in limiting a "cytokine storm" after 920-cGy TBI.

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