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GD3 Ganglioside Antibody Augments Tumoricidal Capacity of Canine Blood Mononuclear Cells by Induction of Interleukin 12[1]

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ABSTRACT

Monoclonal antibody R24 recognizes ganglioside GD3 expressed on the cell surfaces of some tumor cells and on a subset of human T lymphocytes. Binding of R24 to these lymphocytes induces proliferation, cytokine production, and activation of intracellular signaling pathways. In the current report, we investigated expression of gangliosides by canine mononuclear immune cells and studied the ability of antiganglioside antibody to activate these cells using tumor cell killing as a measure of activation. A subset of canine monocytes, but not lymphocytes, was found to express gangliosides GD3 and GD2 as determined by the binding of monoclonal antibodies R24 and 14.G2a, respectively. Only R24 augmented the tumoricidal potential of fresh canine peripheral blood mononuclear cells (PBMCs) against tumor cell lines that did not express surface gangliosides GD3 or GD2. The augmenting effect of R24 on PBMC-mediated tumor cytotoxicity required cooperation between monocytes and lymphocytes because there was no enhancement of cytotoxicity mediated by R24 combined with either monocytes or lymphocytes individually. The enhancing effect of R24 on canine PBMC-mediated tumor cytotoxicity was blocked by anti-interleukin (IL)-12 neutralizing antibody, suggesting that R24 binding to monocytes triggered IL-12 release, contributing to the observed tumor killing effects. Reverse transcription-PCR confirmed that the binding of R24 to canine monocytes induced transcription of mRNA for canine IL-12. These data indicate that monocytes can be activated for tumoricidal responses through a membrane structure associated with ganglioside GD3 triggered by the binding of R24 and that the mechanism for enhanced cytotoxicity is due to the production and secretion of IL-12.

INTRODUCTION

Mabs[3] mediate a number of effector functions useful for cancer immunotherapy. These include ADCC and CDC (1–4). Tumor antigens recognized by Mabs are not always unique to tumor cells. For example, some immune effector cells, such as human lymphocytes, express membrane ganglioside GD3 (5, 6), a glycolipid that is an antigenic determinant found on human tumors arising from neuroectodermal tissue, including melanoma (7, 8). Targeting surface glycolipids on human melanoma cells with anti-GD3 Mabs, such as Mab R24 (IgG3), is an approach that has stimulated clinical interest and has shown promise in the treatment of human melanoma patients (3, 9, 10). Interest has also grown in determining the effects of Mab directed against GD3 on the function of immune cells expressing this antigen (5, 6, 11, 12).

Binding of R24 to normal human lymphocytes induces a number of T-cell activities including proliferation and cytotoxicity (6, 11). Peripheral blood lymphocytes from human melanoma patients receiving R24 also show enhanced proliferative responses after *in vivo* treatment with low doses of R24 (3). R24 ligation of GD3 on human lymphocytes triggers cellular activation, resulting in intracellular tyrosine phosphorylation and ras activation (11, 12). Thus, this antibody demonstrates bifunctional activity by binding tumor cells and thereby eliciting cellular and humoral antitumor responses, on the one hand, and by directly activating lymphocytes after binding to them, on the other.

We previously described a canine malignant melanoma immunotherapy model in which antiganglioside antibodies were shown to bind fresh canine melanoma and mediated ADCC of canine malignant melanoma with canine effectors *in vitro* (13). R24 also triggered CDC of melanoma mediated by canine complement (14). In the current study, we sought to determine whether GD3 is expressed on canine immune cells and, if present, whether R24 would bind these cells, triggering their activation. We found that canine peripheral blood monocytes, but not lymphocytes, expressed GD3, unlike human PBMCs. Binding of R24 to these monocytes enhanced tumor killing mediated by canine PBMCs against GD3-negative canine tumor targets. The mechanism responsible for the enhanced cytotoxicity triggered by R24 acts through the induction of IL-12. These results indicate novel activation of monocytes triggered by antibody binding to a surface ganglioside that may also have clinical relevance in the treatment of spontaneous melanoma in a canine model with R24 antibody.

MATERIALS AND METHODS

Tumor Cells. Malignant canine cell lines included CTAC (15), a gift from Dr. J. Jardine (M. D. Anderson Cancer Center, Houston, TX); malignant canine glioma (clone 5; Ref. 16), a gift from Dr. H. Whelan (Medical College of Wisconsin, Milwaukee, WI); and D17 canine osteosarcoma (American Type Culture Collection, Rockville, MD). These cells were grown as adherent cultures in RPMI 1640 supplemented with 10% v/v heat-inactivated FCS, 2 mM L-glutamine, 10 mM HEPES buffer, penicillin (100 units/ml), and streptomycin (100 μg/ml). Cells were detached with 0.54 mM EDTA for use as single cell suspensions in experiments.

Effector Cells. Canine PBMCs were obtained from heparinized venous blood of 12 healthy adult dogs by separation over a Ficoll-Hypaque (Histopaque-1077; Sigma, St. Louis, MO) discontinuous density gradient (specific gravity, 1.077), as described previously (13, 17). The isolated cells were washed three times in HBSS and resuspended in supplemented RPMI 1640. The viability of the isolated PBMCs, as assessed by trypan blue dye exclusion, was routinely greater than 98% at the time of culture.

Antibodies. Murine Mabs R24 (IgG3 isotype) and 14.G2a (IgG2a isotype), which were kindly provided by the Biological Response Modifiers Program of the National Cancer Institute, recognize gangliosides GD3 and GD2, respectively, expressed on human melanoma cells (2, 7). Tük 4 (murine IgG2a; Dako Corp., Carpinteria, CA) reacts with the CD14 antigen expressed on human monocytes and macrophages and also recognizes CD14 on canine monocytes (18). Antihuman IL-12 polyclonal neutralizing antibody (goat IgG) was purchased from R&D Systems (Minneapolis, MN). An azide-free irrelevant murine IgG3 isotype Mab was purchased from Southern Biotechnology Associates (Birmingham, AL).

Preparation of R24 F(ab′)$_2$ Fragments. F(ab′)$_2$ fragments were prepared by digestion with pepsin using an ImmunoPure F(ab′)$_2$ kit (Pierce, Rockford, IL) according to the manufacturer's instructions. Briefly, 10 mg (4 ml) of R24

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[3] The abbreviations used are: Mab, monoclonal antibody; PBMC, peripheral blood mononuclear cell; ADCC, antibody-dependent cellular cytotoxicity; CDC, complement-dependent cytotoxicity; IL, interleukin; RT-PCR, reverse transcription-PCR; CTAC, canine thyroid adenocarcinoma; LPS, lipopolysaccharide; SAC, *Staphylococcus aureus cowan* strain I; hrIL, human recombinant IL; NK, natural killer.

antibody were dialyzed against a 20 mM sodium acetate buffer (pH 4.5). The sample was then lyophilized, resuspended to 1 ml in digestion buffer [20 mM sodium acetate (pH 4.5)], and added to a tube containing 0.25 ml of immobilized pepsin. The sample was incubated for 1 h at 37°C in a shaking water bath. The solubilized $F(ab')_2$ fragments were recovered using a serum separator and placed on a protein A column equilibrated with binding buffer. The column was washed with binding buffer, and the eluted protein was dialyzed against PBS (pH 7.4). The digested sample was analyzed for the presence of $F(ab')_2$ fragments by SDS-PAGE using sample buffer that did not contain β-mercaptoethanol.

Flow Cytometry. Flow cytometry was used to assess the binding of Mabs R24 and 14.G2a to the CTAC, D17, and malignant canine glioma cell lines using standard indirect immunofluorescence techniques on a FACScan flow cytometer (Becton Dickinson, San Jose, CA). Cells (2×10^5 cells/50 μl) were incubated with 1 μg of Mab for 30 min at 4°C. The cells were then washed with PBS. Goat antimouse IgG conjugated to FITC (Becton Dickinson) was used to detect binding of R24 or 14.G2a by incubation with the tumor cells for 30 min at 4°C in the dark. The cells were washed with PBS, and 5000 cells were analyzed. Irrelevant monoclonal murine IgG3 was used as an isotype control antibody for R24, and an irrelevant mouse IgG2a was used as an isotype control antibody for 14.G2a. Propidium iodide was added just before cell analysis as a live-dead discriminator.

Reactivity of R24 and 14.G2a with canine lymphocytes and monocytes was determined by incubating canine PBMCs (2×10^5 cells/50 μl) with either 1 μg of R24 or 14.G2a. To block nonspecific goat immunoglobulin binding to Fc receptors, the PBMCs were initially incubated with goat IgG for 10 min at 4°C before the addition of R24 or 14.G2a (19, 20). After 30 min, the cells were washed with PBS and incubated with goat antimouse IgG conjugated to FITC for 30 min at 4°C in the dark. The cells were washed with PBS and analyzed on the flow cytometer. Separate gates were established to analyze the monocyte and lymphocyte populations individually determined by forward and side light scatter, as described previously (21). An identical protocol was used to assess the binding of Tük 4 (anti-CD14) to canine monocytes.

Mab R24 $F(ab')_2$ fragments were generated as described above. R24 $F(ab')_2$ fragment reactivity with canine PBMCs was determined by first incubating canine PBMCs (2.5×10^5 cells/50 μl) with approximately 1–5 μg of R24 $F(ab')_2$ fragment protein for 30 min at 4°C. Cells were washed with PBS and subsequently incubated with goat antimouse IgG conjugated to FITC for 30 min at 4°C in the dark. Cells were washed with PBS and analyzed on the flow cytometer. Propidium iodide was added just before cell analysis as a live-dead discriminator. Separate gates were established to analyze monocyte and lymphocyte populations as described for staining with intact R24 antibody. An irrelevant mouse IgG $F(ab')_2$ fragment (Jackson ImmunoResearch Laboratories, West Grove, PA) was used as an isotype control antibody.

Cytotoxicity Assay. A ^{51}Cr release assay was used to assess cytotoxicity of target cell lines, as described previously (13, 22). Target cells (CTAC, D17, and malignant canine glioma cells) were labeled with 250 μCi of ^{51}Cr for 2 h at 37°C in 5% CO_2. These cells were washed in medium, and 5×10^3 cells (in 50 μl of medium) were added to round-bottomed microwells containing serial dilutions of effector cells (also in 50 μl of medium). Viability of target cells (determined by eosin exclusion) before their use in killing assays was greater than 98%. The concentration of effector cells was adjusted to give final E:T ratios of 100:1, 50:1, and 25:1. Some assays were done at E:T ratios of 50:1 or 10:1. Cytotoxicity of ^{51}Cr-labeled target cells was measured in the absence or presence of R24 or 14.G2a, each at a concentration of 10 μg/ml in 50 μl of medium, added just before the addition of target cells. In some experiments, PBMCs were cultured with R24 (50 μg/ml) for 3 days before adding target cells directly to these wells. LPS (25 ng/ml; Sigma) was added to some wells to activate monocytes as a positive control (23, 24). In some assays, antihuman IL-12 polyclonal neutralizing antibody (1 μg/ml) was added along with R24. The final volume of each well was adjusted to 200 μl by adding medium as necessary. All assays were done in quadruplicate. Plates were centrifuged at $500 \times g$ for 5 min and incubated at 37°C for 16 h. We and others have previously shown that a 16-h ^{51}Cr release assay provides reliable cytotoxicity information and may be more informative than a 4-h assay in assessing differences between cytotoxicity mediated by canine PBMCs (25, 26) because the kinetics of cytotoxicity mediated by canine PBMCs progresses at a slower rate compared to human or mouse PBMCs in these assays, and differences in cytotoxicity are not always distinguishable in the 4-h assay with canine effectors. Supernatants were then harvested with a Skatron harvesting system (Skatron, Sterling, VA). Radiolabel released into the supernatant was measured with a gamma counter. The percentage of cytotoxicity was calculated using the following formula:

$$\% \text{ of cytotoxicity} = \frac{\text{Exp. cpm} - \text{Spon. cpm}}{\text{Max. cpm} - \text{Spon. cpm}} \times 100$$

in which Exp. represents the experimental number of counts from target cells incubated with effectors, *Spon.* represents the spontaneously released counts obtained from targets incubated in medium alone, and Max. represents the maximum counts obtained from targets lysed with a 2% centrimide detergent solution (Sigma). Spontaneous release of radiolabel from tumor cells was routinely between 20–30% of maximum release (*e.g.,* 1646 cpm, spontaneous; 6777 cpm, maximum). ^{51}Cr release data from all three serial E:T ratios (*i.e.,* 100:1, 50:1, and 25:1) were also converted to lytic units. One lytic unit is defined as the number of effector cells required to cause 20% lysis of 5×10^3 target cells. Lytic units are expressed as lytic units/10^7 effector cells harvested. In some cytotoxicity assays, tumor cell killing was evaluated at a single E:T ratio because of limited numbers of effector cells that were available after immunomagnetic selection. Results from these experiments are reported as the percentage of cytolysis because a range of E:T ratios is needed to convert the percentage of cytolysis to lytic units. All reagents used in cytotoxicity assays, including R24, were endotoxin-free when assayed by the *Limulus* Amebocyte Lysate test (Pyrotell; Associates of Cape Cod, Inc., Woods Hole, MA).

Immunomagnetic Cell Depletion. A miniMACS Magnetic Separation System (Miltenyi Biotec, Auburn, CA) was used to remove $CD14^+$ canine monocytes from PBMCs according to the manufacturer's instructions. Canine PBMCs obtained from Ficoll-Hypaque separation of whole blood were incubated with Tük 4 antibody (murine anti-CD14; final concentration, 10 μg/ml) in 100 μl of degassed buffer (0.5% BSA in PBS) for 15 min at 6°C. The cells were washed and resuspended in 80 μl of buffer, and 20 μl of colloidal superparamagnetic microbeads (Miltenyi Biotech) conjugated to goat antimouse IgG were added for 15 min at 6°C. The cells were then washed, resuspended in 100 μl of buffer, and put into a miniMACS Separation Column (Miltenyi Biotech) that was suspended on a miniMACS magnet. The effluent cells, depleted of $CD14^+$ monocytes, and the positively selected cells were collected individually and used in cytotoxicity assays. Flow cytometry of the positively selected monocytes and negatively selected PBMCs was used to assess the completeness and purity of the cell separation.

Detection of Canine IL-12 by Immunoblot. Canine PBMCs were isolated as described above and plated in 24-well plates (5×10^6 cells/well) in AIM V serum-free lymphocyte medium (Life Technologies, Inc., Gaithersburg, MD). Cells were stimulated for 40 h with 0.0075% fixed SAC cells (Pansorbin; Calbiochem, La Jolla, CA), the most powerful inducer of IL-12 in human immune cells (27), at which time the supernatants were collected and lyophilized. Each 1-ml sample was resuspended in 200 μl of sample buffer, and the proteins were separated by SDS-PAGE. Samples were transferred to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA). The membrane was blocked using a 5% nonfat dry milk solution in Tris-buffered saline (pH 8.0), followed by incubation with the biotinylated form of the antihuman IL-12 polyclonal neutralizing antibody (R&D Systems; 0.2 μg/ml). Finally, the blot was incubated with streptadvidin linked to alkaline phosphatase (1 μg/ml) and developed using the appropriate substrates in 0.7 M Tris buffer (pH 9.7). hrIL-12 and mouse recombinant IL-12 (R&D Systems) were used as positive controls.

Isolation of RNA and Reverse Transcription. Canine PBMCs were isolated as described above and resuspended in supplemented RPMI 1640 at 2×10^6 cells/ml. Aliquots (2 ml) were placed in 6-well, flat-bottomed tissue culture plates in the presence or absence of Mab R24 (50 μg/ml). A total of 1.2×10^7 cells were seeded for each condition, divided between three wells. After 24, 48, and 72 h, the cells were pelleted, the supernatants were discarded, and 0.5 ml of Trizol reagent (Life Technologies, Inc.) was added to the cell pellet, with an additional 0.5 ml of the reagent also added to each well of the tissue culture plate. Total RNA was then isolated according to the manufacturer's instructions. Reverse transcription was carried out according to the manufacturer's instructions using the Superscript Preamplification System (Life Technologies, Inc.), and equal amounts of RNA were used for each

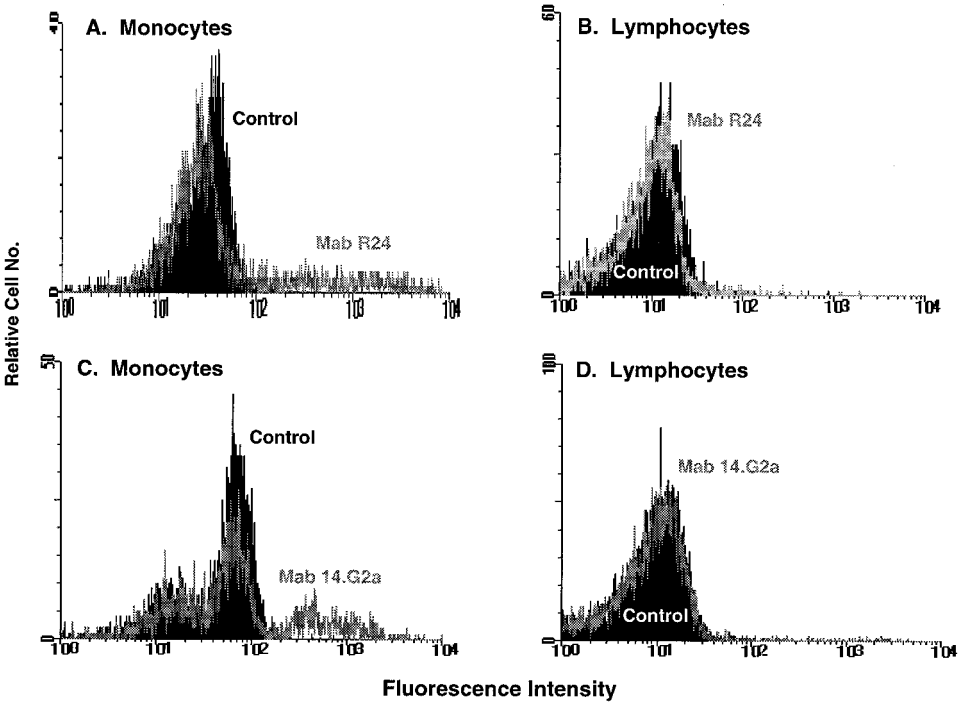

Fig. 1. Expression of gangliosides by canine peripheral blood immune cells as determined by flow cytometry. Indirect flow cytometry was performed to examine canine peripheral blood immune cells for expression of ganglioside GD3 (*A* and *B*) and ganglioside GD2 (*C* and *D*) by binding Mabs R24 and 14.G2a, respectively, to monocytes and lymphocytes. The isotype control antibodies used were irrelevant murine IgG3 for R24 and IgG2a for 14.G2a. Binding of primary antibody was detected with goat antimouse IgG conjugated to FITC. In the *upper histograms*, (*A*) 17.7% of the monocytes (mean fluorescence intensity, 533) and (*B*) 0.7% of the lymphocytes (mean fluorescence intensity, 38) were $R24^+$, whereas for 14.G2a (*lower histograms*), (*C*) 19.7% of the monocytes (mean fluorescence intensity, 583) and (*D*) 0.9% of the lymphocytes (mean fluorescence intensity, 28) were $14.G2a^+$. Data shown are from one dog and are representative of the results obtained from eight healthy canine blood donors.

reaction. As a positive control, RNA was also isolated from PBMCs that were cultured for 18 h with 0.0075% SAC.

Amplification of the IL-12 Subunits by the PCR. Primers directed against the genes of the canine p35 and p40 subunits of IL-12 were used to selectively amplify fragments of cDNA generated from cells cultured with or without R24 for various times. The forward primer (5′-TGTTGTAGAGTTGGACTGGCACC-3′) and the reverse primer (5′-CAATGGCTTCAGCTGCAGGTTTG-3′) for the p40 gene fragment yielded a product of approximately 600 bp, whereas the forward primer (5′-CTGCACTTCCGAAGAGATTGATC-3′) and the reverse primer (5′-AAATCCGGCTCTTCAAGGGAG-3′) for the p35 gene fragment yielded a product of approximately 350 bp. Forward (5′-CATGTTTGAGACCTTCAACACCCC-3′) and reverse (5′-GCCATCTCTTGCTCGAAGTCCAG-3′) primers to β-actin, which was used as a control, yielded a product of approximately 300 bp. The p40 and the p35 fragments were selectively amplified using the following conditions: 94°C for 2 min followed by 35 cycles of 94°C for 30 s, 53°C for 30 s, and 72°C for 30 s. The cycles were followed by a 7-min extension at 72°C. Amplification of the p40 fragment required the presence of 1.5 mM $MgCl_2$, whereas amplification of the p35 subunit required the presence of 3.0 mM $MgCl_2$. The β-actin fragment was amplified under the following conditions: 95°C for 2 min followed by 35 cycles of 95°C for 1 min, 50°C for 1 min, and 72°C for 2 min followed by a 10-min extension at 72°C. Amplification of the product was done in the presence of 1.5 mM $MgCl_2$. Taq polymerase was from Fisher (Itasca, IL) or Promega (Madison, WI).

Statistical Analysis. Differences in the percentage of cytotoxicity and lytic units were assessed using nonparametric paired *t* tests (paired by dog). Calculations were done with the SAS statistical analysis package.

RESULTS

Expression of GD3 and GD2 Gangliosides on Canine Immune Effector Cells and Tumor Targets

PBMCs. Fresh PBMCs obtained from donor dogs were analyzed on a flow cytometer to assess binding of Mabs R24 and 14.G2a to surface GD3 and GD2, respectively. Separate gates for analysis of lymphocytes and monocytes were set based on differences in forward and side light scatter between the two cell types (21). Staining with Tük 4 antibody for the monocyte surface marker CD14 correlated with the monocyte gate established by light scatter. The stained canine monocytes showed bright fluorescence for R24, which bound approximately 18% of the monocytes (Fig. 1*A*). Although variations in the percentage of stained monocytes were observed between dogs (*i.e.*,

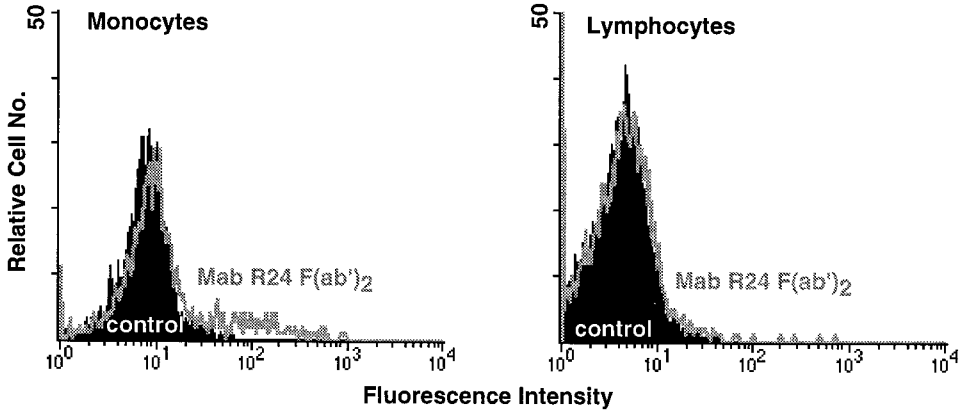

Fig. 2. Recognition of canine monocytes by Mab R24 $F(ab')_2$ fragments. To confirm that binding of the anti-GD3 antibody R24 to canine monocytes was due to specific recognition of monocyte surface GD3 rather than nonspecific binding to Fc receptors, reactivity of $F(ab')_2$ R24 fragments with fresh canine PBMCs was assessed by flow cytometry. Similar to intact antibody, $F(ab')_2$ fragments of R24 specifically stained a subset (15%) of canine monocytes (*left*), whereas binding to lymphocytes was minimal (0.7%; *right*).

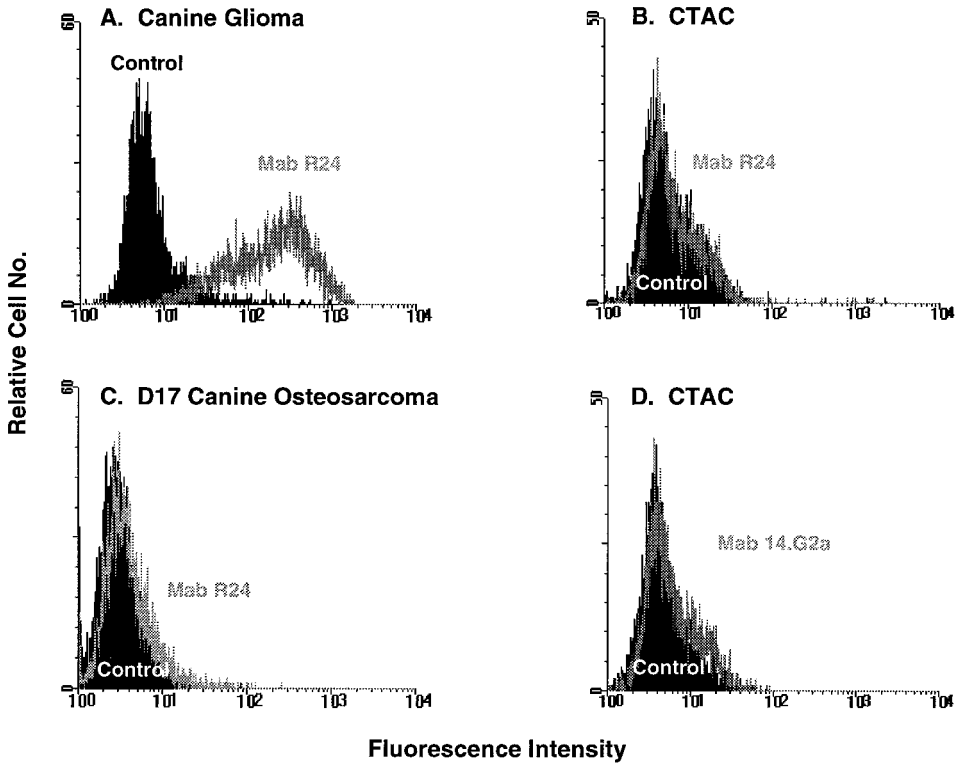

Fig. 3. Expression of disialogangliosides on canine tumor cells as shown by flow cytometry. Indirect flow cytometry was performed to assess expression of ganglioside GD3 on the malignant canine glioma cell line (*A*), CTAC (*B*), and the canine osteosarcoma line D17 (*C*) by the binding of anti-GD3 Mab R24. Expression of ganglioside GD2 on the CTAC cell line was also assessed by the binding of Mab 14G.2a (*D*). The isotype control antibodies were irrelevant murine IgG3 and IgG2a, respectively. Binding of primary antibody was detected with goat antimouse IgG conjugated to FITC.

approximately 15–35% $R24^+$ monocytes), the percentage of $R24^+$ monocytes remained similar for each of 10 individual dogs tested on two or more flow cytometry analyses. In contrast, less than 1% of canine lymphocytes bound R24 (Fig. 1*B*). Similar to R24, Mab 14.G2a bound approximately 20% of the monocytes (Fig. 1*C*) but less than 1% of the canine lymphocytes (Fig. 1*D*).

Flow cytometry also demonstrated that Mab R24 $F(ab')_2$ fragments specifically bound approximately 15% of the gated canine monocytes but minimally recognized canine lymphocytes (0.7%; Fig. 2). These results confirmed that Mab R24 bound canine monocytes by Fab recognition of GD3 expressed on these cells.

Malignant Canine Glioma. R24 showed specific binding to the canine glioma cell line (Fig. 3*A*). The pattern of staining indicated that GD3 was present on most canine glioma cells, suggesting that this cell line could be used for targeted cell-mediated lysis with R24. Mab 14.G2a did not label the canine glioma cell line.

CTAC. Neither R24 nor 14.G2a bound to the CTAC cells, indicating that this carcinoma cell line did not express GD3 or GD2 on its surface (Fig. 3, *B* and *D*).

D17 (Canine Osteosarcoma). Labeling of D17 cells with R24 was negative, indicating a lack of expression of surface GD3 ganglioside (Fig. 3*C*).

Enhancement of Cell-mediated Cytotoxicity of the $GD3^+$ Canine Glioma Target by Mab R24

In 16-h ^{51}Cr release assays, inclusion of the R24 Mab in the assay augmented cytotoxicity of malignant canine glioma cells mediated by fresh canine PBMCs (Fig. 4). This suggested that variable region binding of R24 molecules to GD3 expressed on the glioma target triggered killing by those immune cells with Fc receptors capable of engaging the Fc end of bound antibody. Thus, R24 mediated ADCC with canine effectors, analogous to the activity we previously observed with the other antiganglioside Mab, 14.G2a, and canine effectors against a ganglioside-expressing canine melanoma cell target (13). Alternatively, variable region binding of R24 to canine monocytes present in the PBMCs could have contributed to monocyte activation, in turn enhancing tumor cytotoxicity. However, based on the flow cytometry results, this explanation is less likely because more glioma cells than monocytes would be expected to have bound the Fab end of the antibody, leaving the FcR binding domain situated to engage immune cells mediating ADCC.

Cell-mediated Lysis of the $GD3^-$ CTAC and D17 Targets Is Enhanced by R24

CTAC. To determine whether the interaction of Mab 14.G2a with $GD2^+$ canine monocytes and R24 with $GD3^+$ canine monocytes affected the cytolytic activity mediated by canine PBMCs, the $GD3^-$/

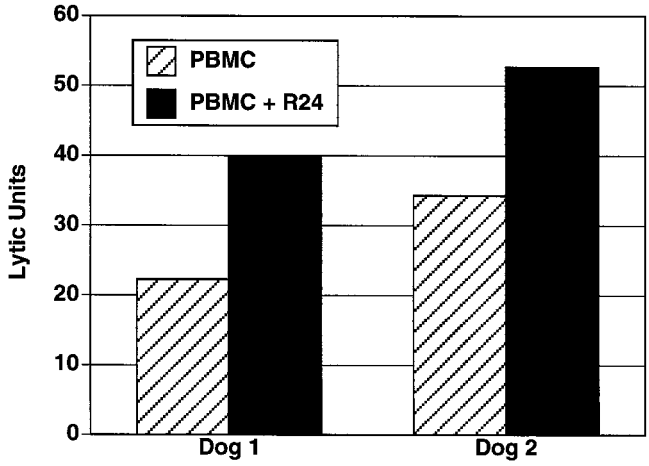

Fig. 4. ADCC mediated by Mab R24 and canine PBMCs. Radiolabeled (^{51}Cr) malignant canine glioma cells were cultured with fresh canine PBMCs without or with R24 (10 μg/ml) for 16 h. R24 augmented canine cell-mediated cytotoxicity of this $GD3^+$ target.

$GD2^-$ CTAC cell line was used as a target. $GD3^-$ D17 cells were also used as targets in some experiments with R24. By using these targets, the conditions needed for ADCC were not provided, ruling out ADCC as a possible mechanism for lysis of the CTAC and D17 targets when either antibody was included. R24 augmented the cytotoxic capacity of canine PBMCs against the $GD3^-$ CTAC target (Fig. 5*A*). Each of eight dogs' PBMCs mediated enhanced cytotoxicity when R24 was present during the 16-h ^{51}Cr release assay compared to cell cultures not containing R24 ($P = 0.014$). The percentage of augmentation varied between individual dogs, with as much as a >2-fold increase observed, with most dogs' PBMCs showing a ≥25% increase in cytotoxicity. The relative augmentation in tumor cytotoxicity induced by R24 was approximately the same for each dog's PBMCs in cytotoxicity assays repeated three times. In contrast, the addition of 14.G2a had no effect on the tumoricidal capacity of PBMCs against the CTAC target (Fig. 5*B*).

D17. R24 significantly ($P = 0.0015$) enhanced the cytotoxicity of canine D17 osteosarcoma cells (Fig. 5*C*). In these experiments, fresh canine PBMCs from five donor dogs were cultured with R24 for 3 days before adding D17 targets to preactivate effector cells, as described previously (11). The concentration of R24 used in the D17 cytotoxicity assays was selected based on the report of Norihisa *et al.* (11) in which 50 μg/ml Mab R24 was shown to be highly stimulatory to the cytotoxic effects mediated by human T cells.

Cell Separation Experiments

When pure populations of canine $CD14^+$ monocytes were positively selected immunomagnetically (Fig. 6) and used as effector cells against the CTAC target, we did not observe augmentation of cytotoxicity with the addition of either R24 or an irrelevant IgG3 isotype control antibody (Fig. 7). Cytotoxicity mediated by the $CD14^-$ cells (*i.e.*, lymphocytes as shown by forward and side light scatter; Fig. 6*C*) plus R24 was also not augmented (Fig. 7). However, when the two populations (*i.e.*, $CD14^+$ and $CD14^-$ cells) were mixed, the R24 enhancing effect was observed (Fig. 7). This suggested that the enhanced cytotoxicity of canine PBMCs stimulated by R24 was due to cooperation between the R24-activated monocytes and lymphocytes present in these cultures.

To control for the possibility that immunomagnetic selection resulted in depressed monocyte responses to activating stimuli, immunomagnetically selected $CD14^+$ monocytes were stimulated with LPS in cytotoxicity assays of the CTAC target (Fig. 8). The LPS-stimulated monocytes that had been immunomagnetically selected mediated enhanced tumor cell killing compared to the unstimulated (immunomagnetically derived) monocytes, suggesting that activation pathways remained intact after the immunomagnetic separation procedure (Fig. 8).

Anti-IL-12 Antibody Blocked R24 Augmentation of Cell-mediated Lysis of the $GD3^-$ CTAC and D17 Targets

The finding that canine monocytes ($CD14^+$ cells) and lymphocytes ($CD14^-$ cells) both needed to be present for R24 to augment tumor cytotoxicity suggested that the binding of R24 to monocytes triggered release of a factor (or factors) that in turn stimulated the cytotoxicity mediated by ($CD14^-$) lymphocytes against the CTAC and D17 targets. One candidate cytokine considered was IL-12, which is secreted by activated monocytes and enhances NK cell-mediated cytotoxicity (28, 29). Moreover, the CTAC line is a NK cell- and lymphokine-activated killer cell-sensitive canine tumor target (25, 30). Therefore, we examined whether an antihuman IL-12 antibody (1 μg/ml) could block the enhanced cytotoxicity of the CTAC and D17 targets mediated by canine PBMCs activated with R24. The addition of anti-IL-12 antibody significantly inhibited lysis of these targets mediated by PBMCs combined with R24 (CTAC, $P = 0.025$; D17, $P = 0.03$), suggesting that enhancement of the cytotoxicity stimulated by R24 binding to canine monocytes could be due, at least in part, to the secretion of IL-12 by these monocytes. Cytotoxicity of the CTAC target mediated by PBMCs alone has previously been attributed to canine NK cell activity (25, 30) and, as expected, was unaffected by the addition of anti-IL-12 neutralizing antibody (data not shown). Similar results were obtained using the D17 target (Fig. 9*A*).

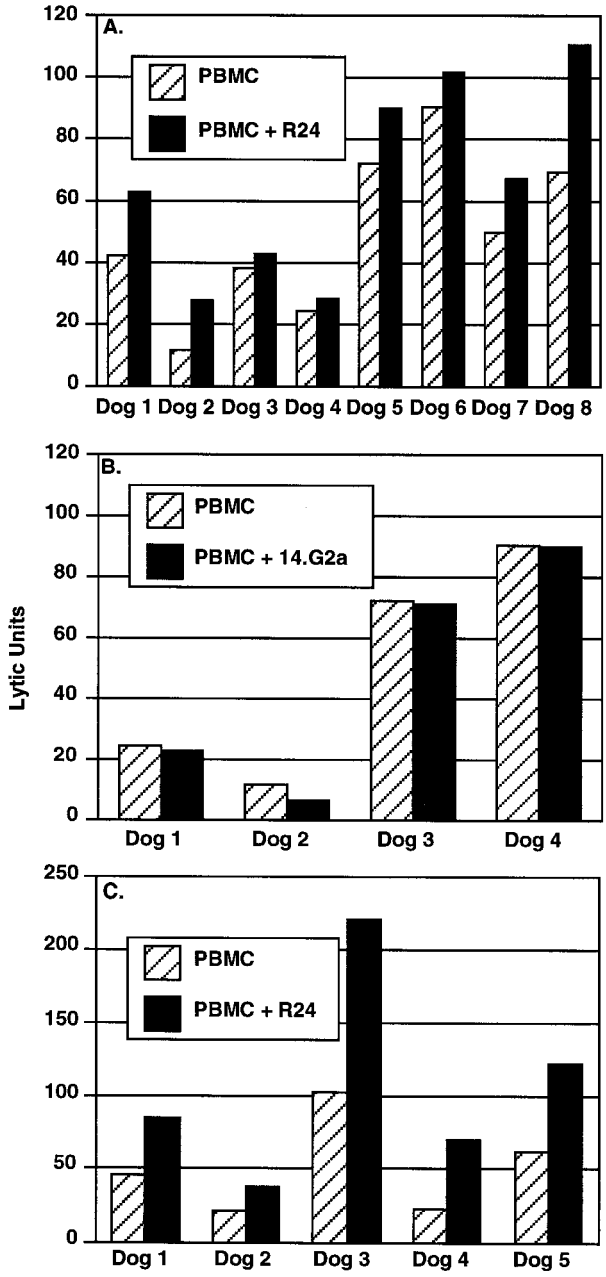

Fig. 5. Mab R24 but not 14.G2a enhanced the cytotoxicity of the ganglioside-negative CTAC and D17 targets mediated by canine PBMCs. *A*, in 16-h ^{51}Cr release assays, the cytotoxicity of $GD3^-$ CTAC cells mediated by canine PBMCs from eight normal dogs was enhanced when R24 (10 μg/ml) was present in the cultures ($P = 0.014$). *B*, the cytotoxicity mediated by fresh canine PBMCs from four dogs against $GD2^-$ CTAC cells was not enhanced in the presence of Mab 14.G2a (10 μg/ml). *C*, PBMCs from five donor dogs were cultured without or with R24 (50 μg/ml) in microwells for 3 days before the addition of D17 osteosarcoma targets in 16-h ^{51}Cr release assays. Cytotoxicity was significantly ($P = 0.0015$) enhanced in cultures containing R24.

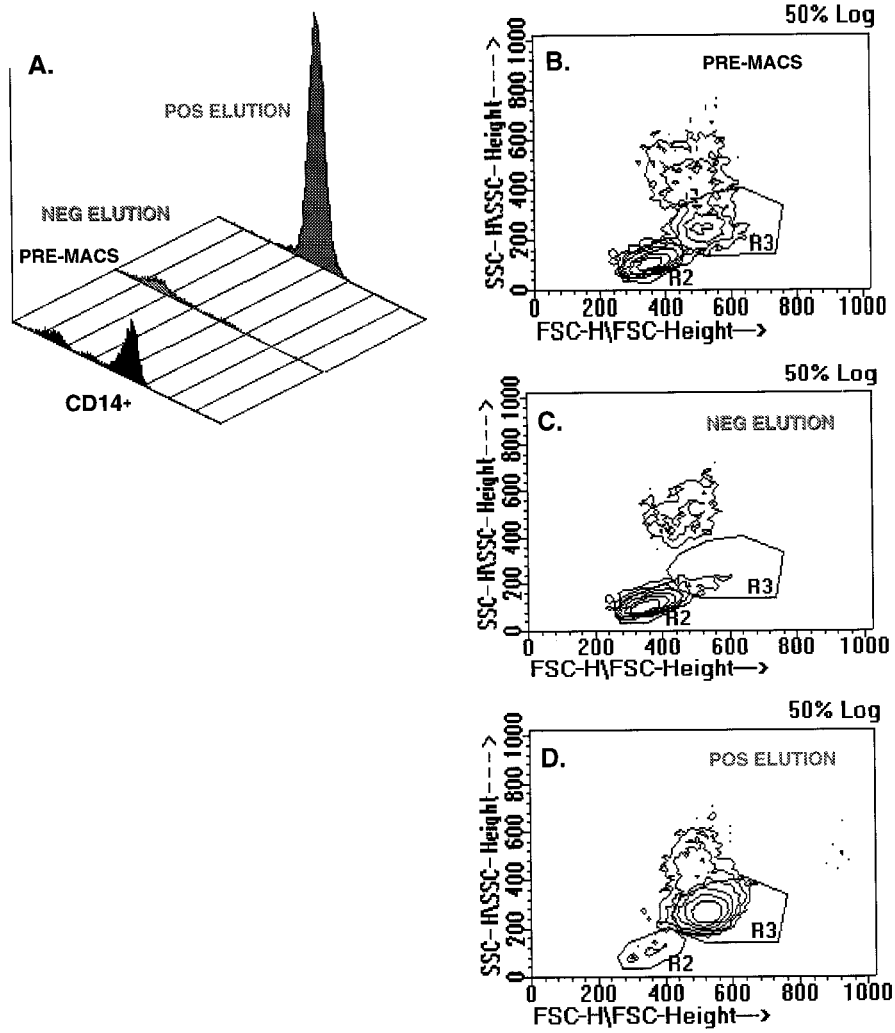

Fig. 6. Selection of CD14$^+$ canine monocytes. Fresh canine PBMCs were incubated with anti-CD14 antibody followed by incubation with goat antimouse IgG conjugated to magnetic beads, as described in "Materials and Methods." The CD14$^+$ monocytes were magnetically selected, and CD14$^-$ cells were collected separately. *A*, indirect immunofluorescence flow cytometry was used to assess CD14 staining of monocytes in the unseparated PBMCs (*PRE-MACS*), CD14$^-$ negatively selected cell (*NEG ELUTION*), and CD14$^+$ positively selected cell (*POS ELUTION*) populations. Forward and side light scatter were used to determine gates for monocytes (*R3*) and lymphocytes (*R2*) in the unseparated PBMCs (*B*) and used for the analysis of CD14$^-$ negatively selected cells (*C*) and CD14$^+$ positively selected cells (*D*). After immunomagnetic separation, the negative elution (*C*) was nearly (>95%) depleted of monocytes, whereas the positive elution (*D*) was nearly (>98%) depleted of lymphocytes.

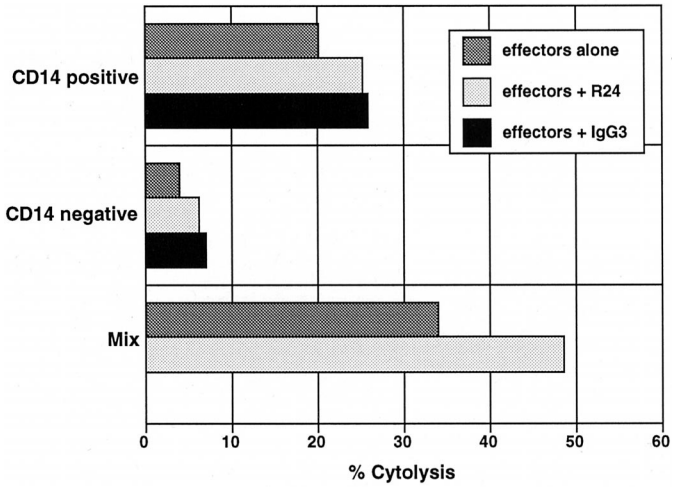

Fig. 7. Cytotoxicity mediated by purified canine CD14$^+$ monocytes and CD14$^-$ immune cells against the CTAC target. Immunomagnetically purified canine monocytes (CD14$^+$) and lymphocytes (*i.e.*, CD14$^-$ cells) were used in cell-mediated lysis experiments of the CTAC target in the absence or presence of R24 (10 μg/ml) at an E:T ratio of 50:1. In some assays, an irrelevant murine IgG3 Mab was used as an isotype control. Cytotoxicity mediated by the combined cell populations (*Mix*) was also evaluated. For the CD14$^+$ and CD14$^-$ effectors, there were no significant differences between the cytotoxicity mediated by effectors alone, effectors plus R24, or effectors plus IgG3. Data shown are representative of four experiments using cells from four dogs at different E:T ratios.

Immunoblotting was performed to verify that the antihuman IL-12 antibody used in these blocking experiments was specifically recognizing canine IL-12 (Fig. 9*B*). Supernatants from canine PBMCs cultured with the IL-12 inducer SAC (27) were analyzed for the presence of IL-12. Bands of the appropriate molecular weights for both subunits of IL-12 were detected, confirming that the antibody does recognize canine IL-12.

Production of IL-12 mRNA Is Induced in Canine PBMCs by R24

As shown in Fig. 9, IL-12 neutralizing antibody abrogated the enhancing effect of R24 on canine PBMC-mediated cytotoxicity, suggesting a mechanistic role for IL-12 in the augmentation of cytotoxicity. RT-PCR was used to determine whether R24 stimulated transcription of IL-12 in bulk populations of canine PBMCs. The rationale for using this approach was based on the failure of commercial human IL-12 ELISA kits to detect canine IL-12 in media obtained from canine PBMCs stimulated with SAC, a potent inducer of IL-12 in other species (data not shown; Ref. 27).

Primers specific for the p35 and p40 subunits of canine IL-12 were used for PCR. RT-PCR performed on RNA extracted from freshly isolated canine PBMCs or PBMCs cultured with R24 indicated that p40 message was not present in fresh PBMCs but was induced in cells cultured with R24, appearing after 1–3 days of incubation (Fig. 10*A*). Cells cultured for 1–3 days without R24 did not show production of

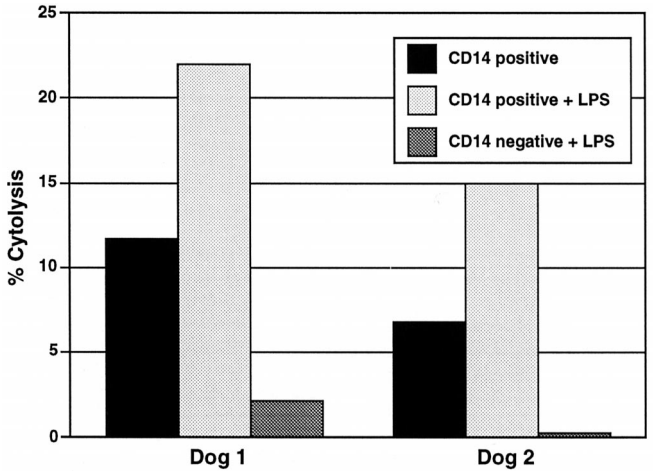

Fig. 8. Activation of immunomagnetically selected CD14$^+$ canine monocytes. CD14$^+$ monocytes (derived by positive immunomagnetic selection) and CD14$^-$ effectors (derived by negative selection) were stimulated with LPS (25 ng/ml) during ^{51}Cr release cell lysis assays to verify that the canine monocytes selected immunomagnetically retained their responsiveness to activating stimuli. The E:T ratio was 10:1.

message for the p40 subunit. On the other hand, p35 message was constitutively expressed in freshly isolated and R24-activated canine PBMCs (Fig. 10*B*). In each case, PCR products were sequenced, and their identities were confirmed as canine IL-12 subunit message. As a positive control, RT-PCR to amplify IL-12 p40 and p35 messages was performed on RNA isolated from canine PBMCs stimulated with SAC for 18 h to induce IL-12 (Fig. 10*C*). These results were identical to those obtained with R24; namely, that the canine p40 subunit is induced by activation with SAC, whereas p35 is constitutively expressed and unchanged by culture with SAC. Expression of IL-12 subunit mRNA in canine PBMCs resembled the murine IL-12 system in which the p40 subunit is also inducible, whereas p35 is constitutively expressed (31). This contrasts with human immune cells, in which the p40 subunit of IL-12 is constitutive, but its basal expression is up-regulated after cell stimulation (27). Transcripts for human p35 are generally present at a low level in most immune cells and are also up-regulated after cell stimulation (27, 32). Thus, as in humans and mice, expression of p40 appears to be highly regulated in the dog.

DISCUSSION

We investigated whether some antiganglioside Mabs used for cancer immunotherapy in humans might recognize and activate tumoricidal pathways in canine immune cells. Our results indicate that R24, an anti-GD3 ganglioside Mab, strongly binds to some canine monocytes and induces heightened antitumor responses triggered by these R24$^+$ monocytes, especially when PBMCs are preactivated with R24 for 3 days. Our findings are consistent with other studies that indicate that the binding of R24 to surface GD3 expressed on human lymphocytes triggers activation pathways when cells are also preactivated with antibody for 3 days (6, 11). However, in contrast to human PBMCs, in the dog, there is a subpopulation of monocytes, not lymphocytes, that express GD3 and are activated by R24. We examined the binding of another antiganglioside Mab, 14.G2a (IgG2a, antiganglioside GD2; Ref. 33), to canine PBMCs, and although this antibody bound to some canine monocytes, it did not enhance PBMC-mediated cytotoxicity against a 14.G2a$^-$ target (CTAC) or induce lymphocyte proliferation (data not shown). Thus, it appears that the augmented tumoricidal function associated with R24 binding to canine monocyte GD3 is a specific effect leading to an activation signal and not a generalized phenomenon associated with antibody binding to

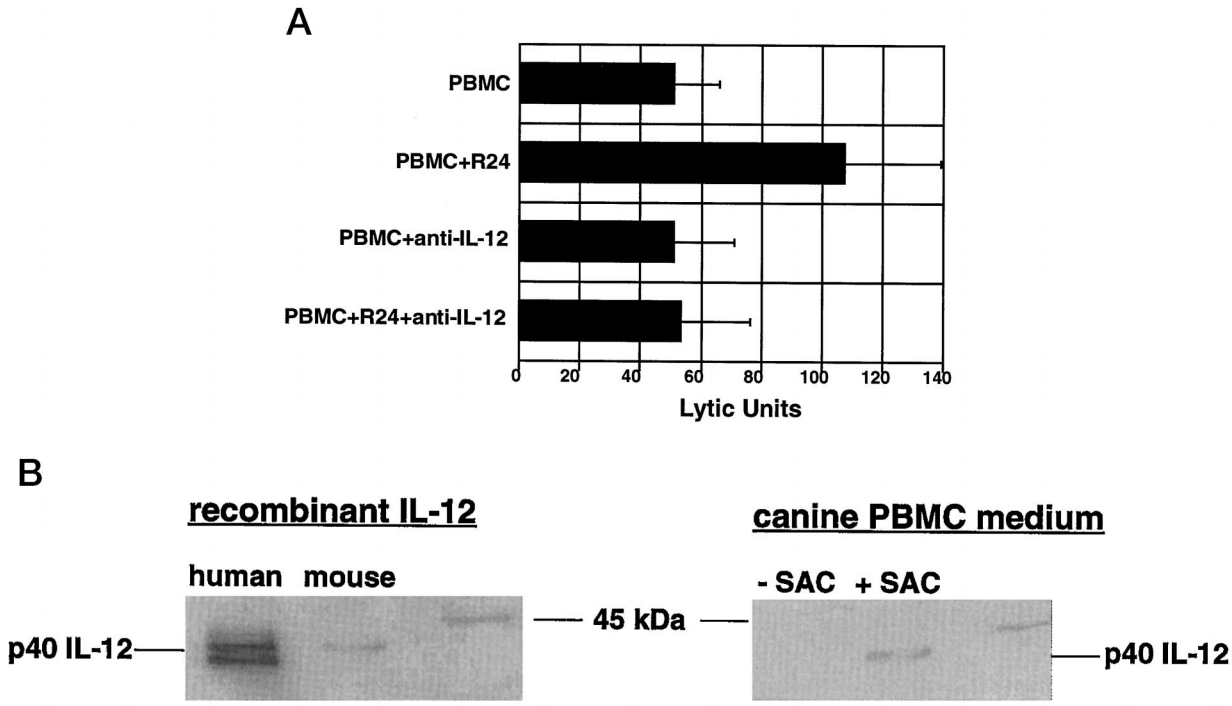

Fig. 9. Anti-IL-12 antibody inhibited cytotoxicity of the GD3$^-$ D17 target mediated by canine PBMCs plus R24. *A*, ^{51}Cr-labeled D17 osteosarcoma cells were used as targets in 16-h cytotoxicity assays in wells containing PBMCs, R24, and anti-IL-12 polyclonal neutralizing antibody (1 μg/ml) that had been in culture for 72 h before the addition of the tumor targets to microwells. Data shown are mean values obtained from five normal dogs. *PBMCs+R24 versus PBMCs+R24+anti-IL-12*, $P = 0.03$. *B*, immunoblotting was performed to verify that the antihuman IL-12 polyclonal antibody used in *A* specifically recognized canine IL-12. The antibody detected the canine p40 subunit of IL-12 in supernatants from canine PBMCs cultured for 48 h with the IL-12 inducer SAC, but not in supernatants from canine PBMCs cultured without SAC. Similar results were obtained using this antibody for detection of the canine IL-12 p35 subunit when compared to human and murine recombinant IL-12 (data not shown). The multiple bands shown for hrIL-12 represent altered glycosylated forms of hrIL-12 routinely detected with this antibody. Fifty ng of hrIL-12 and mouse recombinant IL-12 were loaded, respectively.

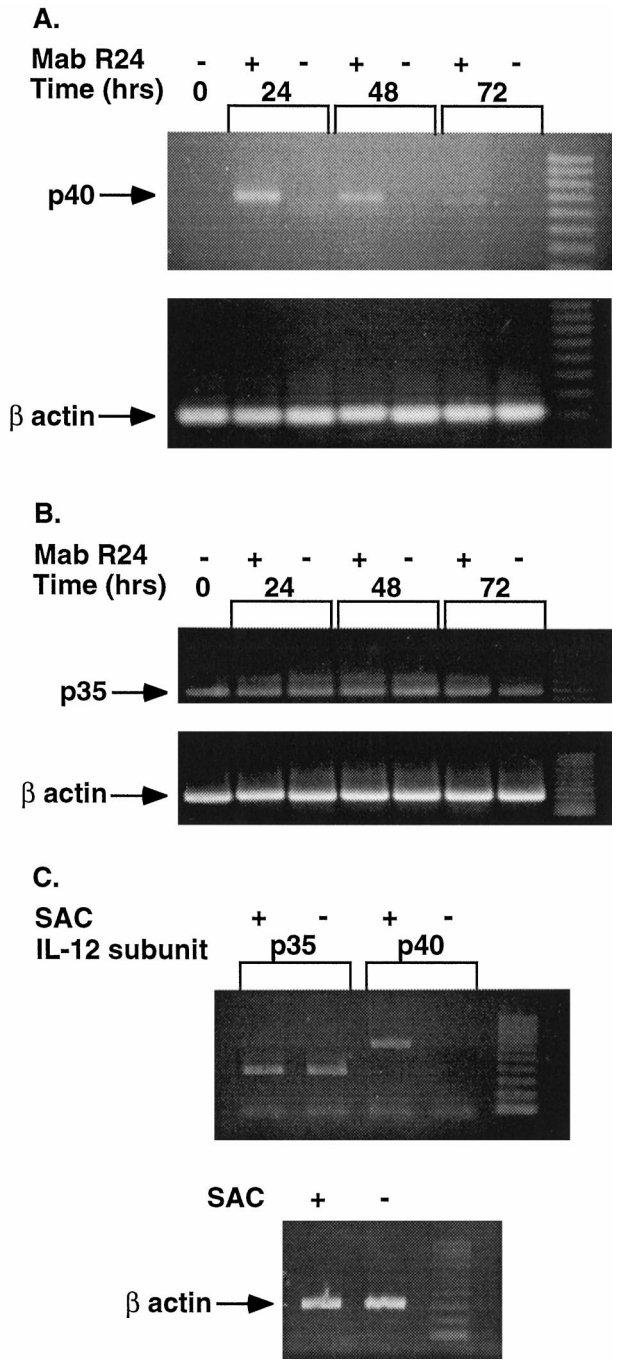

Fig. 10. IL-12 mRNA is induced in canine PBMCs by Mab R24. Freshly isolated canine PBMCs were cultured with Mab R24 for 1–3 days, and then total RNA was isolated as described in "Materials and Methods." An aliquot of RNA was reverse transcribed, and the cDNA was subjected to PCR using primers specific for the p35 and p40 subunits of canine IL-12. Primers were derived from the genomic sequences of canine IL-12 available through GenBank. *A*, PCR analysis of the p40 IL-12 subunit indicates its presence in PBMCs cultured with R24, detectable after 24 h and declining by 72 h in culture, but not in freshly isolated PBMCs (time 0) or PBMCs cultured without R24 for 1–3 days. PCR for β-actin performed on cDNA from these cells is included as a positive control and indicates that approximately equal amounts of cDNA were loaded for each condition examined. Molecular weight markers are present in the lane furthest to the *right*. *B*, RT-PCR of canine PBMCs shows that the p35 message is expressed in freshly isolated PBMCs and is unchanged in cells cultured without or with Mab R24 for 24, 48, or 72 h. Sequencing of the double bands revealed the heavier molecular weight product to be a p35 precursor and the band of the lower molecular weight product to be that of p35. *C*, as a positive control, canine PBMCs were cultured without or with SAC cells for 18 h to induce IL-12. RT-PCR was then performed on the isolated RNA, amplifying for canine IL-12 p35 and p40 subunit cDNA. The p35 message was present in PBMCs cultured with or without SAC cells, whereas the p40 message was present only in the PBMCs stimulated by the SAC cells. Data shown with Mab R24 are from individual dogs in each panel and are representative of experiments performed at different times with PBMCs from four dogs.

other surface gangliosides. These findings are in agreement with those of others (6, 11) that suggest that R24 recognizes a unique epitope of GD3 capable of activating immune cells when specifically bound by antibody. Furthermore, the ability of R24 to enhance canine PBMC-mediated cytotoxicity was demonstrated against two canine tumor cell lines of different histogenesis, suggesting that the stimulating effects of R24 may have broad effects. Alternatively, different effects of the Mabs could also reflect different characteristics of the antibodies relating to isotype and association constants.

Augmentation of cell-mediated cytotoxicity by R24 required the presence of both monocytes ($CD14^+$ cells) and lymphocytes ($CD14^-$ cells) comprising freshly isolated canine PBMCs, whereas individual populations of either $CD14^+$ or $CD14^-$ cells plus R24 failed to mediate enhanced cytotoxicity. This suggested the possibility that R24-stimulated monocytes provided help to activate lymphocytes, thereby resulting in the increases in cytotoxicity that we observed. Stimulation of immune cells often results in cytokine release, and activation of human T lymphocytes through binding of R24 to GD3 expressed on these cells induces the secretion of IFN-γ and, to a lesser degree, tumor necrosis factor α and IL-6 (11). Whereas numerous cytokines could potentially be elaborated from monocytes activated through a pathway triggered by R24 binding to GD3, we considered the possibility that one such molecule was IL-12, due to its stimulatory effects on NK lymphocytes, and the induction of IL-12 is a characteristic of stimulated human and murine monocytes (29). In addition, IL-12 stimulation of lymphocytes could in turn induce additional cytokine release (*e.g.*, IFN-γ) that would augment the tumoricidal capacity of monocytes. An antihuman IL-12 neutralizing antibody significantly abrogated cytotoxicity mediated by canine PBMCs plus R24. There was a great likelihood that this antibody specifically blocked canine IL-12 activity because it is polyclonal, and the high degree of identity between canine and human p40 (87%) and canine and human p35 (88%) suggests that the specificity of such an antibody would be conserved. Furthermore, immunoblotting confirmed that this antibody specifically recognized canine IL-12.

RT-PCR was used to demonstrate that R24 induced transcription of canine IL-12 mRNA in PBMCs. This strategy was necessary because several commercial ELISAs did not detect IL-12 from conditioned medium of cultures containing canine PBMCs plus R24 but did detect IL-12 in medium containing human PBMCs plus the IL-12 inducer SAC. This result was not surprising, given the high species specificity inherent in Mab recognition of epitopes that may differ slightly between species. The addition of R24 to PBMCs stimulated the transcription of the p40 subunit mRNA for IL-12, whereas production of the constitutively produced message for p35 was unchanged upon the addition of R24. Because both subunits of IL-12 are required for its activity, it appears that the mechanism by which R24 augments cytotoxicity of canine PBMCs involves the induction of biologically active IL-12. The induction of other cytokines derived from monocytes is also possible. The p40 subunit of IL-12 was also induced by SAC, suggesting that cellular stimulation with Mab R24 activates similar genes, and is consistent with a shared IL-12 activation pathway by SAC and Mab R24. Production of IL-12 by antigen-presenting cells can occur independently of T cells or can be dependent on the interaction of CD40 on antigen-presenting cells and CD40 ligand expressed on T helper lymphocytes and requires exogenous IFN-γ as an obligatory complementary cosignal (34). It is unlikely that the induction of IL-12 by Mab R24 was mediated through the CD40/CD40 ligand pathway because R24 stimulated IL-12 production without the addition of excess IFN-γ.

We have described a new activity of anti-GD3 antibody R24 on canine monocytes. Throughout these experiments, conditions were

rigidly controlled to rule out effects from contaminating endotoxin or nonspecific binding of the antibody to canine monocytes. We also demonstrated the possibility that R24 mediated ADCC with canine effector cells against an $R24^+$ canine glioma tumor target, although conclusive proof of this activity would require experiments with anticanine Fc blocking antibodies that are not yet available. In addition, we have previously shown that R24 mediated CDC with canine complement to lyse $R24^+$ tumor targets (14). Taken together, these results suggest that R24 exhibits bifunctional activity in the dog, mediating cytotoxicity of $GD3^+$ tumor cells through cellular and humoral mechanisms and specific activation of a subset of canine monocytes. The mechanism for canine monocyte activation by R24 appears to be novel through a GD3-triggered pathway, resulting in IL-12 secretion. The $R24^+$ canine monocytes represent a minority of the circulating canine monocyte pool, comprising about 4–6% of the PBMCs. It is therefore remarkable that this small percentage of cells exerted a measurable increase in cytotoxicity mediated by PBMCs, which suggests that $R24^+$ canine monocytes could be important in modulating cytotoxic responses of canine PBMCs.

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