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Induction of Therapeutic T-Cell Immunity by Tumor Targeting with Soluble Recombinant B7-Immunoglobulin Costimulatory Molecules[1]

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ABSTRACT

Tumor targeting with immunomodulatory molecules is an attractive strategy to enhance the host's antitumor response. Expression of CD80 (B7-1) and CD86 (B7-2) costimulatory molecules in tumor cells has proven to be an efficient way to enhance their immunogenicity. Here, we studied the effects of tumor targeting with biotinylated recombinant soluble B7-1- and B7-2 immunoglobulin G molecules (bio-B7-IgG) using a pretargeting approach based on the sequential use of a biotinylated antitumor monoclonal antibody and avidin. Mouse RMA T-lymphoma cells bearing either bio-B7-1-IgG or bio-B7-2-IgG on their surface prime *in vitro* naive $CD8^+$ CTLs, which are highly effective in adoptive immunotherapy, and induce therapeutic immunity when injected in tumor-bearing animals. *In vivo* targeting of established RMA tumors with bio-B7-IgG either cures tumor-bearing mice or significantly prolongs their survival. The antitumor response induced by targeted bio-B7-IgG depends on both $CD4^+$ and $CD8^+$ T cells. Moreover, tumor targeting with bio-B7-IgG *in vivo* is critical for both expansion in lymphoid organs and mobilization into the tumor of tumor-specific $CD8^+$ CTLs. When targeting is performed on poorly immunogenic TS/A mammary adenocarcinoma, only bio-B7-1-IgG primes naive CTLs *in vitro* and cures or significantly prolongs the survival of tumor-bearing mice *in vivo*, confirming that the two costimulatory molecules are not redundant with this tumor. Altogether, these data suggest that tumor avidination and targeting with soluble bio-B7-IgG may represent a promising strategy to enhance the antitumor response in the host.

INTRODUCTION

Tumor cells are antigenic and can be recognized by autologous tumor-specific T cells (1). These findings provide a strong rationale for the application of immunotherapy to cancer, aimed at activating the potential antitumor T-cell repertoire present in each patient.

For optimal induction, T cells require two activating signals (2). Signal one is generated by the interaction of the TCRs[4] with their cognate peptide-MHC complex. Signal two is induced through the binding of particular T-cell-activating molecules with their counterreceptors, called costimulatory molecules. The best characterized costimulatory pairs are the B7-1 and B7-2 molecules, which are expressed mainly by professional antigen-presenting cells, and their T-cell counterreceptors CD28 and CTLA4 (2–4). The lack of B7 expression by tumor cells may partially explain why they often escape the host's immune system (2, 5). Indeed, experimental tumors, which are sufficiently antigenic, become immunogenic when transfected with B7-1 or B7-2 genes and are rejected by syngeneic animals (6–10). The main effect of the expression of B7 on tumor cells is the activation of a specific T-cell response (6–10). Moreover, it has also been shown that the expression of B7 on tumors activates the innate arm of the immune system, which plays a major role in the rejection of some tumors (11–14). Most of these data have been generated with tumors transduced with the genes coding for B7-1 or B7-2. We have devised an alternative approach that can more easily generate tumor cells bearing the extracellular domain of B7 on their surface. Moreover, this approach can target the expression of B7 costimulatory molecules on tumor cells *in vivo*, without requiring the transduction of tumor cells with the genes encoding the costimulatory molecules. This approach is based on the sequential incubation of cultured tumor cells with biotinylated antibodies specific for a membrane TAA, avidin, and biotinylated soluble B7-1 or B7-2 fused with the IgG constant regions (bio-B7-IgG). We show that targeting mouse tumor cells with bio-B7-IgG *in vitro* is an efficient way to expand tumor-specific CTLs for adoptive immunotherapy and to generate therapeutic nonreplicating whole cell vaccines. More importantly, *in vivo* treatment of tumor-bearing animals with bio-B7-IgG induces a therapeutic T-cell immunity.

MATERIALS AND METHODS

Mice. BALB/c and C57BL/6 4–8-week-old female mice were purchased from Charles River Laboratories (Calco, Italy).

Tumor Cell Lines. The Rauscher virus-induced RMA T-lymphoma of C57BL/6 origin ($H\text{-}2^b$) and the spontaneously arisen, moderately differentiated TS/A mammary adenocarcinoma of BALB/c origin ($H\text{-}2^d$) were maintained *in vitro* in complete medium (RPMI 1640 with 5% FCS, 100 units/ml penicillin, 100 units/ml streptomycin, and 2.5×10^{-5} M 2-mercaptoethanol). RMA cells express the endogenous Thy 1.2 antigen, whereas TS/A cells do not.

Thy 1.1 Cloning and Transfection. Both the cloning of mouse Thy 1.1 cDNA and the generation of RMA cells expressing the Thy 1.1 antigen on their surface (RMA-T cells) have been described previously (15). TS/A cells expressing the Thy 1.1 antigen (TS/A-T cells) were obtained following the same protocols.

Generation of Recombinant Soluble B7-Ig. The ectodomain of human B7-1 was obtained from full-length cDNA (11) by PCR using the following oligonucleotides: (*a*) 5′-CCTGAGCTCCTGAAGCCATGGGCCACACACGG-3′, 5′ primer; and (*b*) 5′-TCTGACTTACCATCAGGAAAATGCTCTTGCTT-3, 3′ primer. The extracellular region of human B7-2 was cloned from full-length cDNA (10) by PCR using the following oligonucleotides: (*a*) 5′-GATATGAGCTCACAGCAGAAGCAG-3′, 5′ primer; and (*b*) 5′-ACTTACCTGAGCTCTGGGGGAGG-3′, 3′ primer. B7-1 and B7-2 cDNAs were cloned into the mammalian expression vectors pCD4-Hγ1 and pCD4-Hγ3, respectively (Ref. 16; kindly obtained from Dr. K. Karjalainen, Basel Institute for Immunology, Basel, Switzerland), replacing the human CD4 ectodomain sequence. The resulting plasmids (p-hB7-1-IgG and p-hB7-2-IgG) were used to transfect mouse J558L plasmacytoma cells by protoplast fusion (16). Transfected cells were selected by growing them in culture medium containing 5 μg/ml mycophenolic acid and 100 μg/ml xantine and screening them for the secretion of B7-IgG soluble chimeric proteins by ELISA. Positive clones were subcloned and expanded to produce the soluble protein. The B7-IgG molecules were purified from culture supernatants by protein G-Sepharose affinity chromatography.

Preparation of bio-19E12 mAb and bio-B7-IgG. Biotinylation of the anti-Thy 1.1 mAb 19E12 was performed as described previously (15). To

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[4] The abbreviations used are: TCR, T-cell receptor; TAA, tumor-associated antigens; NAV, neutravidin; NWSC, nylon wool spleen cells; Ig, immunoglobulin; mAb, monoclonal antibody; IL, interleukin; hrIL, human recombinant IL.

produce 19E12 F(ab′)$_2$ fragments, 2 mg of mAb were mixed with 5 mg of pepsin in 6 ml of 0.1 M sodium citrate (pH 4.5) and incubated for 2 h at 37°C. After dialysis against 1.5 M glycine and 3 M sodium chloride, the Fab fragments were separated from Fc-containing molecules by protein A-Sepharose affinity chromatography. The final product consisted of 90% of F(ab′)$_2$ fragments with minor contamination by Fab fragments, as judged from SDS-PAGE analysis.

Biotinylation of B7-Ig was achieved by mixing 0.5 mg of the recombinant molecule at 1 mg/ml of 0.1 M sodium bicarbonate (pH 8.5) with 6 mg of sulfo-NSH-LC-biotin (Pierce Chemical Co., Rockford, IL) at 1 mg/ml in water. After 30 min at 25°C, the reaction was stopped by dialyzing against PBS. The endotoxin content of all of the biotinylated molecules used for this study was determined by the *Limulus* amebocyte lysate Pyrogent (BioWhittaker, Walkersville, MD) to be ≤0.06 EU/ml.

***In Vitro* Targeting of Tumor Cells with bio-B7-IgG.** RMA-T or TS/A-T cells (1×10^6 cells/ml) were mixed with 1 μg of bio-19E12 mAb in 50 μl of PBS containing 2% FCS (PBS/FCS) and incubated for 10 min on ice. After washing, the cells were incubated for 10 min on ice with 1 μg of NAV (a deglycosylated avidin with a neutral isoelectric point; Pierce Chemical Co.) in 50 μl of PBS/FCS, washed again, and finally resuspended in 50 μl of PBS/FCS containing 3 μg of bio-B7-Ig. After 10 min on ice, the cells were washed and used for the subsequent experiments.

***In Vitro* Priming of Tumor-specific CTLs.** *In vitro* priming of tumor-specific CTLs was performed according to our published protocol (11). CD8$^+$ T cells (80–90% pure) were further purified from NWSCs by complement-mediated lysis of CD4$^+$ T cells using the anti-CD4 mAb GK1.5 and rabbit complement (LowTox Cedarline). Nonreplicating tumor cells were generated by incubating 10^7 cells with 100 μg of mitomycin C in 1 ml of medium for 45 min at 37°C. CTLs were generated by culturing $10–20 \times 10^6$ CD8$^+$ T cells with $2–4 \times 10^6$ nonreplicating RMA-T cells bearing bio-B7-IgG or bio-IgG in a 6-well plate containing 5 ml of complete medium. After 48 h of culture, hrIL-2 was added at 10 units/ml (Hofman-LaRoche, Basel, Switzerland). When using nonreplicating TS/A-T cells bearing bio-B7-IgG or bio-IgG to prime naive CTLs, total spleen cells were used. After 48 h, hrIL-2 and hrIL-7 (Boheringer Mannheim, Penzberg, Germany) were added at 10 units/ml each. In the inhibition experiments, either purified anti-CD8 mAb 53.6.72, human CTLA-4 IgG1, anti-MHC class I K^b mAb Y3, or anti-D^b mAb B22.249 was added at the beginning of the cocultures at a final concentration of 1 μg/ml. Long-term antitumor CTL lines were maintained by restimulating T cells every 7–10 days with nonreplicating RMA-T cells prepulsed with bio-B7-IgG in the presence of 20 units/ml hrIL-2. To generate an alloreactive CTL line, $10–20 \times 10^6$ NWSCs from a naive C57BL/6 mouse were cultured with $2–4 \times 10^6$ nonreplicating J558L plasmacytoma cells of BALB/c origin and expanded as described above.

Cytotoxicity Assays. Blasts at day 6 of culture were tested in a standard 4-h ^{51}Cr release assay (11). ^{51}Cr spontaneous release was always <25% of the maximal (1 N HCl) ^{51}Cr release. The percentage of specific ^{51}Cr release was calculated as follows: (experimental cpm − spontaneous cpm)/(maximum cpm − spontaneous cpm) × 100.

Cytofluorimetric Studies. The presence of bio-B7-Ig on tumor cells after *in vitro* or *in vivo* targeting (see below) was assessed by cell staining with FITC-conjugated goat antihuman IgG antiserum (Southern Biothec., Birmingham, AL). The phenotype of spleen cells was determined by tricolor cytofluorimetric analysis using FITC-labeled rat antimouse CD8α mAb, R-phycoerythrin-labeled rat antimouse CD69L mAb or R-phycoerythrin-labeled rat antimouse CD44 mAb, and biotinylated mouse antimouse Vβ5.1–5.2 TCR mAb, followed by Cychrome-conjugated streptavidin (all reagents were from PharMingen). Analysis was performed using a FACScan cytofluorimeter (Becton Dickinson, Palo Alto, CA).

***In Vivo* Studies.** All *in vivo* studies were approved by the Ethical Committee of the Istituto Scientifico San Raffaele and performed according to its guidelines. To evaluate the therapeutic efficacy of nonreplicating RMA-T cells bearing bio-B7-IgG, 3×10^4 living parental RMA cells were injected s.c. into the left flank of B6 mice. Three days later, these mice began the therapeutic regimen, which consisted of six s.c. contralateral injections of 1×10^6 mitomycin C-treated RMA-T cells bearing bio-B7-IgG, administered twice a week for 3 weeks.

In adoptive transfer experiments, 3×10^4 living parental RMA cells were injected s.c. into mice. After 72 h, 10^6 anti-RMA CTLs were injected i.p. into mice, together with hrIL-2 (2000 units) given once a day for the next 3 days.

For *in vivo* targeting experiments, mice bearing either a 24-h or a 72-h-old RMA-T tumor generated by the s.c. injection of 3×10^4 living cells received 50 μg of bio-19E12 mAb F(ab′)$_2$ fragments i.p., followed 24 h later by NAV (30 μg; i.p.) and 6 h later by bio-B7-IgG or bio-IgG (50 μg; i.p). For the TS/A-T tumor model, 4×10^4 living cells were injected s.c., followed 48 h later by the i.p. injection of 50 μg of bio-19E12 mAb (whole mAb). NAV and bio-B7-IgG molecules were than injected i.p. 24 h and 6 h, respectively, after the anti-Thy 1.1 mAb.

For *in vivo* depletion experiments, 300 μg of either purified rat IgG, anti-CD4 GK1.5, or anti-CD8 53-6-72 mAb were injected i.p. into mice 48 h and 24 h before the tumor inoculum. This regimen causes the depletion of more than 90% of target lymphocytes from both the spleen and lymph nodes. An injection of the same amount of antibody was given once a week until the completion of the experiment.

To assess the development of a memory response, 25–30 days after the primary inoculum, tumor-free mice were challenged contralaterally with 10^5 RMA or TS/A parental cells and scored for tumor growth.

Immunohistochemistry Analysis of Tumor Sections. Animals bearing a 24-h-old RMA-T tumor underwent the three-step targeting protocol. After 13 days, tumors were excised, fixed with 4% paraformaldehyde, embedded in OCT (Miles, Inc., Elkhart, IN), and frozen in liquid nitrogen. Cryostat sections (10 μm) were stained with FITC-labeled anti-CD4 and anti-CD8 mAb (PharMingen).

Statistical Analysis. All experiments performed *in vivo* were repeated at least twice with groups of 5–10 mice, giving homogeneous results. Survival curve data were accumulated and analyzed using the log-rank statistic test.

RESULTS

Tumor Cells Bearing bio-B7-IgG Prime Specific CTLs *in Vitro*. The efficacy of the costimulatory signal provided by targeted bio-B7-IgG molecules was first evaluated *in vitro* using RMA lymphoma cells bearing soluble B7-IgG molecules to prime tumor-specific naive CTLs. This tumor was chosen because it induces antitumor T cells when transfected with either full-length B7-1 or B7-2 cDNA (10). To enable specific binding of B7-IgG to tumor cells, we exploited a three-step-dependent strategy based on sequential incubations with: (*a*) biotin-conjugated mAb specific for a membrane TAA; (*b*) avidin; and (*c*) biotin-conjugated B7-IgG. To generate a surface TAA selectively expressed by tumor cells, RMA cells were transfected with the cDNA encoding the Thy 1.1 allele, which is not expressed by C57BL/6 mice. Thy 1.1 is poorly immunogenic in C57BL/6 mice and does not alter the growth rate of transfected tumor cells either *in vitro* or *in vivo* (data not shown). Bio-B7-IgG molecules bound to RMA-T cells with the three-step approach were stable on the surface of the tumor cells for at least 24 h at 37°C, indicating that the complexes are not internalized or are poorly internalized (data not shown). Moreover, we determined that both bio-B7-1-IgG and bio-B7-2-IgG were functional because they bind Chinese hamster ovary cells expressing transfected CTLA-4 on the surface and costimulate IL-2 production in a mouse T-cell hybridoma activated by a suboptimal concentration of anti-CD3 mAb (data not shown).

The results of coculture experiments with NWSCs from naive animals showed that RMA-T cells coated with either bio-B7-1-IgG or bio-B7-2-IgG indeed prime tumor-specific CTLs *in vitro* (Fig. 1*A*). The addition of either soluble CTLA4-Ig, anti-CD8 mAb, or anti-D^b mAb, but not rat IgG, during the coculture abolished the priming (Fig. 1*B*). Thus, the B7 ectodomain of the conjugate and the D^b-peptide complexes on RMA as well as the expression of CD8 on CTLs were all necessary for the priming.

Adoptive Immunotherapy of Tumor-bearing Mice with CTLs Primed *in Vitro* by RMA-T Cells Coated with bio-B7-IgG. We next assessed whether RMA-T cells coated with bio-B7-IgG could

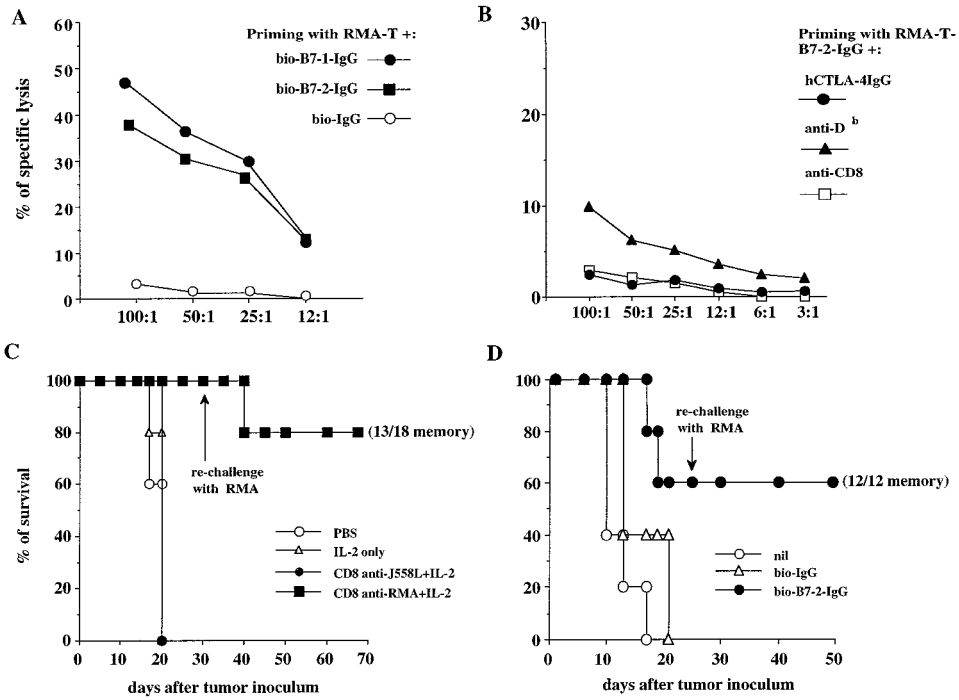

Fig. 1. Antitumor responses induced by RMA tumor cells targeted with bio-B7-IgGG molecules *in vitro*. *A*, RMA-T cells bearing bio-B7-IgG prime specific $CD8^+$ CTLs *in vitro*. Nonreplicating RMA-T cells were pulsed with bio-B7-IgG molecules as described in "Materials and Methods" and incubated with purified naive $CD8^+$ T cells. T-cell blasts were assayed 6 days later for cytolytic activity using Cr^{51}-labeled nontransfected RMA. Nonspecific killing was measured using RMA-S cells as targets and did not exceed that induced by RMA cells bearing bio-IgG. *B*, molecular requirements for *in vitro* priming of naive $CD8^+$ T cells by RMA-T cells bearing bio-B7-2-IgG. Purified CTLA4-Ig, anti-D^b mAb, or anti-CD8 mAb was added to the priming cultures described above. Cytolytic activity of T-cell blasts was assayed after 6 days of culture. Nonspecific killing of RMA-S targets was 5% at a 100:1 E:T ratio. *C*, adoptive immunotherapy with tumor-specific $CD8^+$ CTLs expanded *in vitro* by RMA-T cells bearing bio-B7-Ig molecules. CTLs were restimulated 10 days after *in vitro* priming using RMA-T bearing bio-B7-2-IgG cells. After 8 days, 1×10^6 CTLs, together with IL-2, were injected i.p. into every mouse bearing a 72-h-old RMA tumor as described in "Materials and Methods." Alloreactive CTLs were induced and expanded *in vitro* using nonreplicating BALB/c-derived ($H\text{-}2^d$) J558L plasmacytoma cells. Mice that remained tumor free 30 days after CTL transfer were rechallenged contralaterally with 10^5 living RMA cells and assessed for the development of a protective response. *D*, nonreplicating RMA-T cells bearing bio-B7-IgG molecules are efficient therapeutic vaccines in tumor-bearing mice. One million nonreplicating RMA-T cells bearing bio-B7-2-IgG were injected s.c. in the right flank of mice bearing a 72-h-old tumor generated by injecting 3×10^4 RMA cells s.c. into the left flank. The therapeutic regimen consisted of two injections per week for 3 weeks. Mice that remained tumor free for 25 days were rechallenged contralaterally with 10^5 living RMA cells and assessed for the development of a protective response.

also efficiently expand tumor-specific CTLs *in vitro*, suitable for adoptive immunotherapy. Ten days after *in vitro* priming, CTLs were restimulated with RMA-T cells coated with bio-B7-IgG molecules and allowed to expand for an additional 8 days in the presence of IL-2. CTLs underwent a 3-fold expansion when restimulated by RMA-T cells coated with bio-B7-IgG and not when restimulated with bio-IgG. At day 8 after restimulation, 10^6 CTLs were transferred into every mouse bearing a 72-h-old RMA tumor generated by the s.c. injection of 3×10^4 living cells. As shown in Fig. 1*C*, all 20 mice receiving the adoptive transfer of anti-RMA CTLs rejected the primary tumor. Moreover, 80% of the cured mice rejected a subsequent contralateral challenge with 1×10^5 living RMA cells, indicating that a systemic memory response was induced (Fig. 1*C*).

Active Immunotherapy with Nonreplicating RMA-T Cells Bearing bio-B7-Ig. As a second therapeutic approach based on the *in vitro* targeting of bio-B7-IgG molecules, we evaluated the use of nonreplicating RMA-T cells coated with bio-B7-IgG as whole cell vaccines. One million nonreplicating RMA-T cells coated with bio-B7-2-IgG were injected s.c. into syngeneic C57BL/6 mice bearing a 3-day-old contralateral s.c. RMA tumor. As shown in Fig. 1*D*, 60% of the mice were cured by this treatment. All of the cured mice developed a memory response, as indicated by the rejection of a subsequent contralateral challenge with 10^5 living parental RMA cells. Nonreplicating tumor cells bearing soluble bio-B7-IgG on their surface can therefore function as a whole cell vaccine for active immunotherapy.

Immunotherapy by Targeting Established RMA-T Tumors with bio-B7-IgG. The therapeutic efficacy of targeting bio-B7-IgG to tumors by the three-step approach was evaluated *in vivo*. In these experiments, all of the targeting compounds (antibody, avidin, and bio-B7-IgG) were administered i.p. The administration of avidin 24 h after the injection of anti-Thy 1.1 biotinylated mAb causes rapid clearance of the antibody from the circulation, as determined by ELISA on the sera of treated animals (data not shown). Moreover, cytofluorimetric analysis of *ex vivo* lymphoma cells showed that bio-B7-2-IgG was still present on the tumor 24 h after complete treatment, but not when avidin was omitted (Fig. 2*A*). These results show that tumor pretargeting with antibody/avidin complexes allows specific and persistent homing of bio-B7-IgG on tumors.

To assess the therapeutic efficacy of the three-step targeting regimen *in vivo*, mice underwent the treatment at different times after receiving a s.c. inoculum of 3×10^4 living RMA-T cells. As shown in Fig. 2*B*, when the treatment started 1 day after the tumor inoculum, 40% and 60% of the mice treated with bio-B7-1-IgG and bio-B7-2-IgG, respectively, rejected the primary tumor. Tumor rejection required the administration of all of the components of the three-step approach because omitting either biotin-anti-Thy 1.1 mAb or avidin or both before injecting bio-B7-2-IgG did not result in any therapeutic effects, suggesting that bio-B7-IgG molecules ought to be targeted onto the tumor (data not shown). All of the cured mice also rejected a secondary contralateral challenge with parental RMA cells, indicating that a long-lasting protective immunity was induced (data not shown). When the three-step-mediated treatment with bio-B7-2-IgG was carried out 3 days after the primary tumor inoculum, the mice developed a tumor, although there was a significantly delay (Fig. 2*C*). Thus, in the case of RMA lymphoma, the therapeutic response appears to be dependent on the tumor burden.

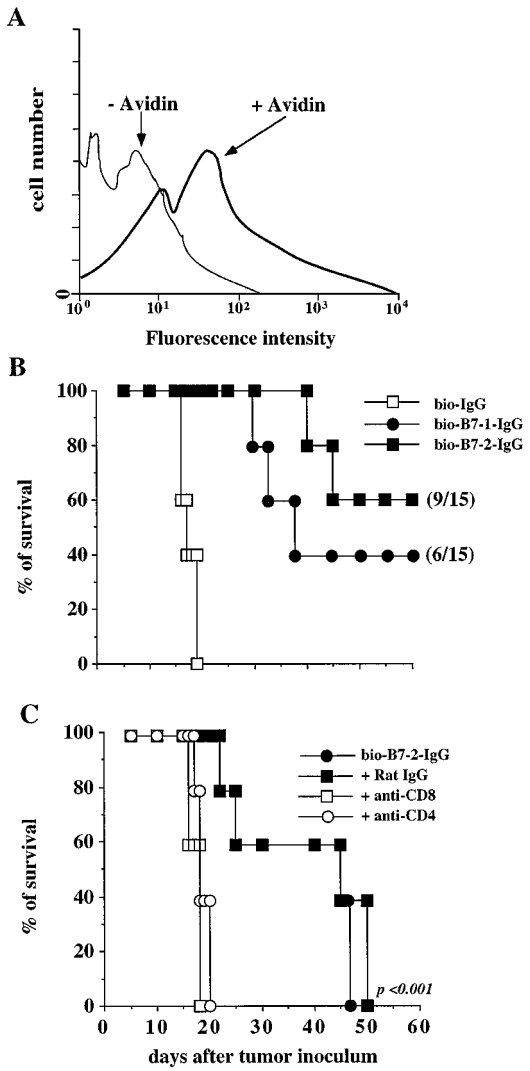

Fig. 2. Therapeutic effects of the *in vivo* targeting of RMA tumor cells with bio-B7-IgG molecules. *A*, efficacy of *in vivo* tumor targeting with bio-B7-IgG by the three-step strategy. One million living RMA-T cells were injected s.c. into B6 mice, and 24 h later, the mice were injected i.p. with 50 μg of bio-19E12 mAb [F(ab′)$_2$], followed 18 h later by 50 μg of NAV, and 6 h later by 50 μg of bio-B7-2-IgG. Animals were killed 24 h after bio-B7–2-IgG injection to excise the tumor mass, which was minced to a single cell suspension and immediately analyzed by flow cytometry for the presence of B7-2-IgG molecules bound to the tumor cells. Histograms indicating the amount of human B7-IgG molecules targeted to tumor cells with or without NAV are shown. One of two reproducible experiments is shown. *B*, treatment of RMA-T tumors by *in vivo* targeting with bio-B7-IgG. Mice were challenged s.c. with 3×10^4 living RMA-T cells, followed 24 h later by the i.p. injection of bio-19E12 anti-Thy 1.1 mAb [F(ab′)$_2$; first step]. NAV was injected 24 h after bio-19E12 (second step), followed 6 h later by either bio-B7-1-IgG or bio-B7-2-IgG (third step). Mice were then scored for tumor growth and sacrificed when the mean tumor diameter reached 10 mm. *C*, both CD4$^+$ and CD8$^+$ T cells play a critical role in the antitumor response induced by *in vivo* targeted bio-B7-2-IgG. Two days and 1 day before the s.c. injection of 3×10^4 living RMA-T cells, mice were treated i.p. with either control rat IgG, anti-CD4, or anti-CD8 mAb. Injection of the mAbs continued once a week throughout the experiment. The three-step protocol started 72 h after the tumor inoculum. Mice were scored for tumor growth and sacrificed when the mean tumor diameter reached 10 mm. The survival distribution curves of mice receiving only control bio-IgG or bio-B7-2-IgG plus either depleting mAb were comparable. *P* refers to the increased survival of mice treated with only bio-B7-2-IgG compared to that of mice treated with bio-B7-2-IgG and anti-CD8 or anti-CD4 mAb.

Tumor Targeting with bio-B7-IgG Molecules Enhances Both Expansion in the Spleen and Mobilization into the Tumor of CD8$^+$ T Cells. To determine the contribution of T cells to the antitumor response induced by *in vivo* tumor targeting with bio-B7-IgG, either CD8$^+$ or CD4$^+$ T cells were depleted from mice before starting the three-step treatment. As shown in Fig. 2*C*, depletion of either subset abolished the therapeutic effect of bio-B7-2-IgG on RMA tumors, indicating that both CD4$^+$ and CD8$^+$ T cells are critically involved in the antitumor response induced by targeting. Mice undergoing *in vivo* tumor targeting with bio-B7-IgG were also evaluated for the development of a tumor-specific CTL response. As shown in Fig. 3*A*, only mice that received bio-B7-2-IgG developed a consistent RMA-specific CTL response after the *in vitro* restimulation of splenic T cells. Of note, both mice from the bio-B7-IgG-treated group did not show a measurable s.c. tumor when sacrificed, whereas both mice from the bio-IgG-treated group displayed macroscopic s.c. tumors (mouse 1 had a larger tumor than mouse 2).

Because the Rauscher virus-derived gag-Leader immunodominant epitope presented by RMA cells on D^b is mainly recognized by CD8$^+$ CTLs expressing the TCR Vβ5.2 family (17, 18), we also investigated whether the *in vivo* targeting of RMA-T tumor with biotin-B7-2-Ig caused a sizeable expansion of primed CD8$^+$ Vβ5$^+$ T cells. *In vivo* targeting of bio-B7-IgG caused a 2- to 3-fold expansion of CD8$^+$ TCR Vβ5$^+$ splenic T cells expressing the antigen-primed markers CD44^{high} (Fig. 3*B*) or CD62L^{low} (data not shown). No significant expansions within CD8$^+$ CD44^{high} T cells expressing control TCR Vβ6, Vβ8, and Vβ13 was observed, indicating that CD8$^+$ T cells expressing TCR Vβ5 were selectively expanded (data not shown).

We finally determined whether *in vivo* targeting of tumor with

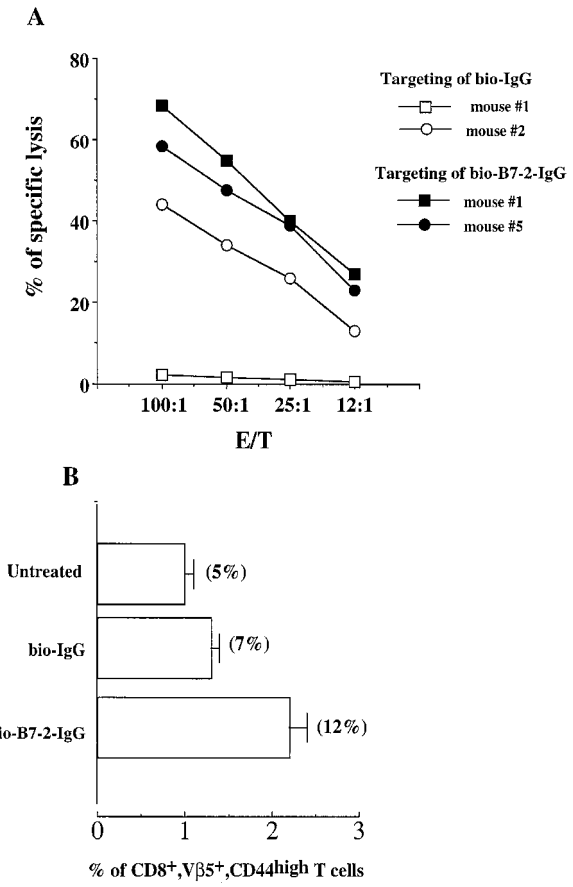

Fig. 3. *In vivo* targeting of RMA-T cells with bio-B7-2-IgG expands RMA-specific CTLs. *A*, induction of RMA-specific CTLs by the *in vivo* targeting strategy. Mice bearing a 24-h-old RMA tumor were subjected to the bio-B7-IgG or bio-IgG targeting protocol. After 10 days, splenic T cells were purified from two mice per experimental condition and restimulated *in vitro* with parental RMA cells. The cytolytic activity of these CTLs was determined 6 days later against ^{51}Cr-labeled RMA cells. *B*, *in vivo* tumor targeting with bio-B7-2-IgG expands RMA-specific CD8$^+$ Vβ5$^+$ CD44^{high} T cells. Spleen cells obtained from mice 10 days after starting the targeting protocol described in *B* were stained with anti-CD8, anti-Vβ5, and anti-CD44 mAb and analyzed by fluorescence-activated cell sorting. The percentage of Vβ5$^+$ CD44^{high} CD8$^+$ T cells in naive, bio-IgG-, and bio-B7–2-IgG-treated mice is shown in *parentheses*. Three mice per group were analyzed.

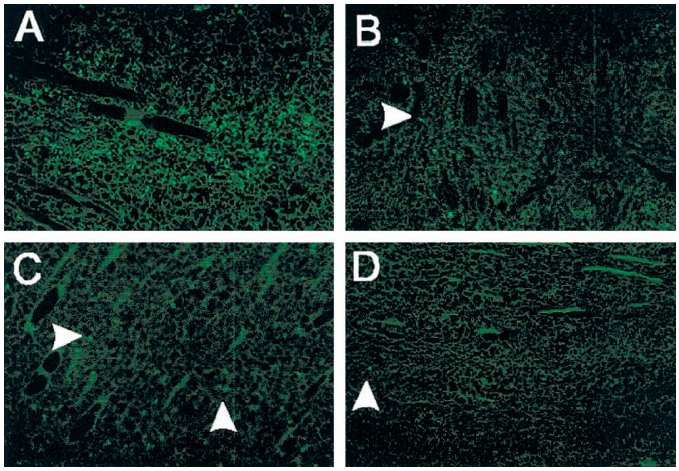

Fig. 4. Tumor targeting with bio-B7-IgG enhances intratumor infiltration by $CD8^+$ T cells. Mice bearing a 24-h-old RMA-T tumor underwent the three-step tumor targeting procedure with bio-B7-2-IgG (*A* and *C*) or bio-IgG (*B* and *D*). After 12 days (corresponding to 15 from the tumor inoculum), tumors were excised, cut in sections (10 μm), and stained with FITC-conjugated anti-CD8 (*A* and *B*) or anti-CD4 (*C* and *D*) mAbs. *Arrows* indicate representative positive T cells.

bio-B7-IgG enhanced intratumor infiltration by T cells. The immunohistochemical analysis of RMA-T tumors excised 13 days after injecting bio-B7-IgG into mice revealed the presence of a marked $CD8^+$ T cells and, although to a lower extent, $CD4^+$ T cell infiltration (Fig. 4, *A* and *C*). The control treatment with bio-IgG did not significantly enhanced intratumor infiltration by $CD8^+$ (Fig. 4*B*) and $CD4^+$ T cells (Fig. 4*D*), giving a picture comparable to that of untreated wild-type RMA tumors (data not shown). Collectively, these results indicate that *in vivo* tumor targeting with soluble bio-B7-IgG costimulatory molecules enhances the endogenous antitumor immunity by determining a selective expansion of tumor-specific CTLs in the spleen as well as by enhancing T-cell infiltration into the tumor.

bio-B7-1-IgG but not bio-B7-2-IgG Is Effective when the Targeting Is Performed on Poorly Immunogenic TS/A Mammary Adenocarcinoma. We determined the efficacy of targeting bio-B7-IgG molecules on a second tumor, the poorly immunogenic TS/A mammary adenocarcinoma. At variance with RMA-T cells, TS/A-T cells coated with bio-B7-1-IgG, but not with bio-B7-2-IgG, primed specific CTLs *in vitro* (Fig. 5*A*). In these experiments, TS/A-T cells were covered with a comparable amount of each of the bio-B7-IgG molecules, as determined by cytofluorimetric analysis (data not shown). In keeping with the *in vitro* data, targeting established TS/A-T adenocarcinoma *in vivo* with bio-B7-1-IgG, but not with bio-B7-2-IgG, induced a 10% tumor rejection with a significant overall delay in the tumor growth (Fig. 5*B*). Thus, at variance with the results obtained with the RMA model, only bio-B7-1-IgG induced an immune response when three-step-mediated targeting was performed with the TS/A mammary adenocarcinoma.

DISCUSSION

We have shown that targeting tumors with recombinant B7-Ig costimulatory molecules by the three-step approach is an efficient strategy to induce and expand therapeutic tumor-specific T cells both *in vitro* and *in vivo*. Targeted soluble bio-B7-IgG and transfected full-length molecules appear to be fully comparable from the functional point of view (11).[5] However, the three-step procedure may overcome the technical limitations that genetic engineering still shows in the generation of tumor vaccines expressing B7. The three-step approach is in fact rapid and simple and does not require the generation of cell lines from each tumor. Primary tumor material from surgical specimens could be armed by the three-step method with bio-B7-IgG molecules, irradiated, and injected back into patients within hours. Moreover, we have also demonstrated that direct tumor targeting by systemic injection of biotinylated antibody, avidin, and bio-B7-IgG conjugate induces a therapeutic immunity against established tumors. The immune activation observed with this treatment was likely dependent on the binding of bio-B7-IgG to tumor cells because no response was observed when either biotin-anti-Thy 1.1 or avidin or both were omitted in control experiments. The relevance of this finding relies on the fact that the treatment was carried out entirely *in vivo*, without previous manipulation of tumor cells *in vitro*.

Depletion experiments indicate that both $CD4^+$ and $CD8^+$ T cells are critically involved in tumor rejection induced by *in vivo* targeted B7-IgG molecules, although the depletion regimen used does not allow us to determine whether either subset is required during the priming and effector phase of the antitumor response. It is clear from this data, however, that targeted B7-IgG cannot bypass the requirement for $CD4^+$ T cell help to induce antitumor $CD8^+$ CTLs. Because

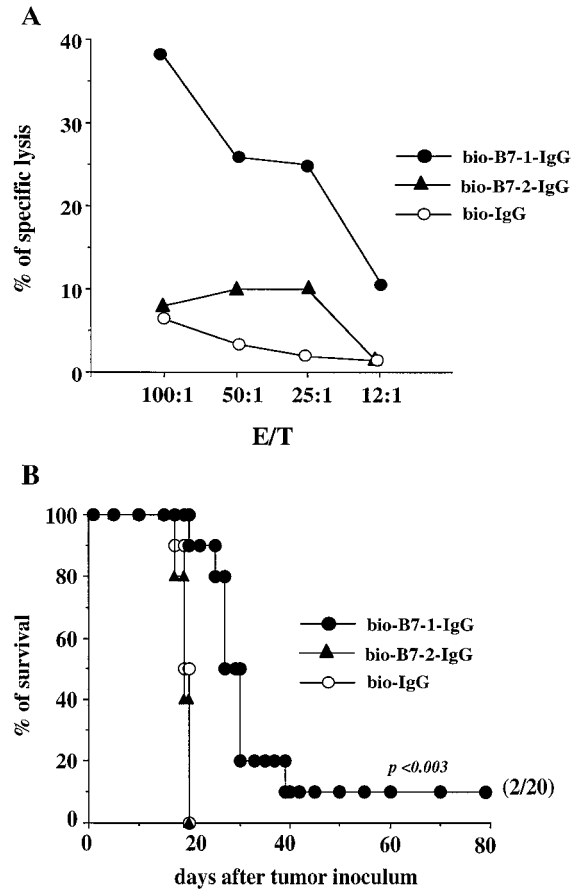

Fig. 5. Bio-B7-1-IgG but not bio-B7-2-IgG is effective when targeting is performed on poorly immunogenic TS/A mammary adenocarcinoma. *A*, poorly immunogenic TS/A mammary adenocarcinoma bearing bio-B7-1-IgG but not bio-B7-2-IgG prime naive CTLs *in vitro*. Total spleen cells from naive BALB/c mice were cultured with nonreplicating TS/A-T cells bearing bio-B7-1-IgG, bio-B7-2-IgG, or bio-IgG, as described in "Materials and Methods." Cytotoxic activity was measured after 6 days. Nonspecific killing measured on the RMA-S target cells was comparable to that induced by TS/A-T cells bearing bio-IgG. One representative experiment of three experiments is shown. *B*, therapeutic efficacy of the three-step-dependent *in vivo* targeting of TS/A-T tumors with bio-B7-IgG. The three-step protocol started 48 h after the s.c. inoculum of 4×10^4 living TS/A-T cells. Mice were scored for tumor growth and sacrificed when the mean tumor diameter reached 10 mm. *P* refers to the increased survival of mice treated with bio-B7-1-IgG compared to that of mice treated with either bio-B7-2-IgG or bio-IgG.

[5] A. Martin-Fontecha, unpublished results.

the RMA tumor used in this experiment cannot express MHC class II molecules, this result suggests an important role for endogenous MHC class II^+ APCs in capturing, processing, and representing TAAs to $CD4^+$ T cells. Whether bio-B7-IgG molecules enhance the cross-priming of tumor-specific $CD4^+$ and $CD8^+$ T cells by endogenous APC or allow for direct activation of tumor-specific $CD8^+$ T cells by targeted tumor cells remains to be established. Targeted bio-B7-IgG most likely enhances both direct presentation and cross-priming of TAA, as recently shown for the full-length B7-1 molecule transfected into TS/A adenocarcinoma (19).

RMA tumor cells are intrinsically immunogenic (10), therefore it is not surprising that they can induce tumor-specific CTLs in some animals in the absence of targeted bio-B7-IgG. However, these T cells are mostly confined in the secondary lymphoid organs and do not efficiently infiltrate the tumor. Accordingly, the CTLs induced by parental RMA tumors do not have therapeutic efficacy. In contrast, targeting RMA tumors *in vivo* with bio-B7-IgG results in two major effects on the host's immune system: (*a*) it markedly enhances the expansion of RMA-specific $CD8^+$ $V\beta5^+$ CTLs in the spleen of all of the mice tested; and (*b*) it induces the mobilization of $CD8^+$ into the tumor and, less prominently, the $CD4^+$ T cells. Conceivably, these two effects are sequentially related, thus the intratumor infiltration by T cells would reflect the induction of an efficient systemic antitumor immunity by targeted bio-B7-IgG. It is also possible, however, that the targeted costimulatory molecules exert some of their effects locally, at the tumor site. For instance, it has been shown that B7/CD28 interaction reduces the apoptosis of T cells during both the induction and effector phases of the immune response (4, 20–22). Thus, targeted B7-IgG may rescue tumor-infiltrating lymphocytes from dying after killing tumor cells, resulting in an improved effector phase at the tumor site. Furthermore, B7-dependent engagement of CD28 at the tumor site may enhance the local production of pro-inflammatory cytokines, favoring the induction of an inflammatory reaction critical for the progression of the antitumor response.

The antitumor mechanisms triggered by the B7 targeting were sufficient to control the growth of only relatively small RMA tumors, suggesting that tumor targeting with soluble B7-IgG molecules would therefore be better aimed at the treatment of minimal residual tumor disease. One possible explanation for this phenomenon is that in the case of large tumors, the CTL expansion is still insufficient to achieve the proper CTL:tumor cell ratio necessary to efficiently kill all of the neoplastic cells. Alternatively, the antitumor response is suppressed progressively in the host upon RMA growth *in vivo*. However, experiments performed with immunogenic tumors showing the presence of an intact immune system in mice bearing a large tumor burden do not support this hypothesis (23).

Whereas both B7-IgG molecules induce efficient antitumor responses when targeted on RMA cells both *in vitro* and *in vivo*, only B7-1-IgG works with TS/A cells under the same conditions. The different effect of B7-1 and B7-2 on the two tumor cell lines is intriguing. We can exclude that is is due to particular features of the soluble chimeric molecules because the B7-IgG molecules used with RMA and TS/A tumors were derived from the same batches. Moreover, the *in vitro* priming data were obtained using either RMA-T or TS/A-T cells covered with a comparable amount of each bio-B7-IgG molecule, thus excluding major quantitative differences between the two soluble costimulatory molecules during priming. Hence, it is tempting to speculate that intrinsic biological differences between B7-1 and B7-2 may explain their unequal effects on RMA and TS/A cells. Several studies performed with other tumor models have underscored the nonredundancy of B7-1- and B7-2-dependent costimulation, usually revealing the superior efficacy of B7-1 (24–26). These studies, together with our results, would suggest the use of B7-1 as a universal costimulatory molecule, although B7-2 could be more efficient with some tumors.

The three-step-dependent targeting strategy used in this study offers several alternative advantages over the use of conventional immunoconjugates: (*a*) rapid circulation clearance of the biotinylated mAb by avidin injected as the second step, which improves tumor:background ratios (27, 28); (*b*) modularity, which allows the use of differential doses of targeting and effector components; and (*c*) an easy combination of different biotinylated antitumor mAbs as well as biotinylated effector molecules, avoiding the requirement to generate all of the corresponding bispecific molecules, each carrying the desired tumor specificity and effector arm. In this respect, a number of mAbs specific for different surface molecules whose expression is restricted to tumors are already available for clinical applications and could be used to target soluble B7 costimulatory molecules *in vivo* (29–31). Moreover, the feasibility of tumor avidination in patients is well documented, and it is currently used in nuclear medicine to increase the uptake and localization of radioactive-labeled biotin for either diagnostic or therapeutic purposes (27, 28, 32–36).

Tumor pretargeting with avidin can also be exploited to deliver other biotin-conjugated effectors to the tumor site. In particular, we found that the pretargeting system can increase the amount and the persistence of biotin-TNF on tumor cells and that cell-bound TNF can trigger antitumor responses *in vivo* (15, 37). Experiments are under way to assess whether a combination of *in vivo* targeting of soluble recombinant B7 and cytokines results in a stronger antitumor response. Moreover, it will also be interesting to determine whether the association between active vaccination with TAA and *in vivo* tumor targeting with B7-IgG can improve the antitumor response.

Collectively, the results presented in this study suggest that tumor targeting with soluble recombinant B7 costimulatory molecules may represent a promising strategy to enhance the host's antitumor immune response.

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