

β -Glucan Functions as an Adjuvant for Monoclonal Antibody Immunotherapy by Recruiting Tumoricidal Granulocytes as Killer Cells

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ABSTRACT

The tumor-killing mechanisms available to monoclonal antibodies (mAbs; e.g., antagonism of growth factor receptors, antibody-dependent cell-mediated cytotoxicity) limit efficacy. Previous studies suggested that i.v. β -glucan might function as an adjuvant for antitumor mAbs. β -Glucan had been shown to function via the iC3b-receptor complement receptor 3 (CR3; CD11b/CD18) thereby enhancing leukocyte killing of tumor cells coated with iC3b via naturally occurring antitumor antibodies. Therapy with β -glucans was limited by levels of natural antibodies and by tumor escape through elimination of antigen-positive cells. Accordingly, it was hypothesized that β -glucan responses could be improved by combined administration with antitumor mAbs. Five tumor models were explored in BALB/c or C57Bl/6 mice using tumors that expressed either high levels of naturally occurring antigens (e.g., G_{D2} ganglioside) or recombinant human MUC1. In comparison with antitumor mAb or β -glucan alone, combined treatment with mAb plus β -glucan produced significantly greater tumor regression in all models that included mammary, s.c., and hepatic tumors. Tumor-free survival only occurred in models that incorporated stable expression of the target antigen. β -Glucan enhancement of the mAb tumoricidal response did not occur in mice deficient in either leukocyte CR3 (CD11b^{-/-}) or serum C3, confirming the requirement for CR3 on leukocytes and iC3b on tumors. Granulocytes appeared to be primarily responsible for tumoricidal activity, because β -glucan therapeutic responses did not occur in granulocyte-depleted mice. These data suggest that the therapeutic efficacy of mAbs known to activate complement (e.g., Herceptin, Rituxan, and Erbitux) could be significantly enhanced if they were combined with β -glucan.

INTRODUCTION

Tumor immunotherapy with humanized monoclonal antibodies (mAbs) such as Herceptin (trastuzumab) and Rituxan (rituximab) is now accepted clinical practice in patients with Her-2/neu⁺ metastatic mammary carcinoma and B-cell lymphoma, respectively (1–6). Based on their record of success, several other humanized mAbs are being developed, and some, such as Erbitux (cetuximab), are apparently close to achieving final Food and Drug Administration approval (7, 8). Nevertheless, antibody therapy is not uniformly effective, even in patients whose tumors express a high surface density of the target tumor antigen. Effector mechanisms thought to cause tumor regression are variable and particularly include inhibition of growth factor activity, as well as antibody-dependent cell-mediated cytotoxicity. Complement-dependent cytotoxicity (CDC) has less frequently been identified as an effector mechanism, and it remains somewhat con-

troversial whether CDC contributes significantly to tumor regression. *In vitro* studies have shown that CDC is limited by membrane regulators of the complement system, such as CD55 and CD59, that are occasionally overexpressed on tumors (9). Moreover, the major complement-mediated effector mechanism used against microbial pathogens, C3-receptor-dependent phagocytosis, and cytotoxic degranulation is completely inactive against cancer (10, 11). With antitumor human IgG1-based mAbs that activate complement such as trastuzumab, rituximab, or cetuximab, a coating of iC3b is deposited on tumor cells that can be recognized by the leukocyte iC3b-receptor complement receptor 3 (CR3; Mac-1, CD11b/CD18, or $\alpha_M\beta_2$ -integrin). However, the triggering of CR3-dependent leukocyte [neutrophil, monocyte, macrophage, and natural killer (NK) cell]-mediated cytotoxicity requires that CR3 bind to both iC3b and a microbial polysaccharide such as β -glucan (12). Because tumor cells do not express CR3-activating polysaccharides, they escape this protective mechanism effective against microbial pathogens.

Fungal β -glucans are well-known biological response modifiers that have been used in cancer patients with varying and unpredictable success, particularly in Japan (13–18). *In vitro* studies demonstrated that soluble yeast β -glucan bound to a lectin domain within the COOH-terminal region of the CD11b subunit of CR3 and that this binding served to prime the receptor for triggering cytotoxic degranulation in response to iC3b-coated tumor cells (10, 19, 20). The presence of small soluble molecules of β -glucan bound to the CR3 of circulating leukocytes apparently allows these cells to recognize and kill iC3b-coated cancer cells in the same way as they kill iC3b-coated yeast cells. Mice genetically deficient in C3 or CR3 (CD11b^{-/-}) have been particularly important in demonstrating the roles of C3 and CR3 in various immune and infectious disease models (21–25) and were especially important in the initial studies of the mechanisms involved in β -glucan therapy involving natural antitumor antibodies (20, 26). Antitumor antibodies that activate complement are required to deposit iC3b on tumor cells, and such deposition of iC3b frequently occurs with tumor cells that are completely resistant to CDC. Thus, β -glucan could potentially be used as an adjuvant for mAb therapy of cancer to provide a leukocyte CR3-dependent mechanism of tumor killing that is additive to all other effector mechanisms.

Previous *in vitro* studies demonstrated that human and mouse monocyte/macrophages, neutrophils, and NK cells could each carry out β -glucan-mediated CR3-dependent cellular cytotoxicity against iC3b-opsonized tumor cells (10, 11, 20). However, the specific effector cell(s) required in mouse tumor models was unknown. Several reports have demonstrated a role for human or mouse granulocytes in tumor rejection (27–29), and because the major leukocyte type identified in Ptas64 tumors was the granulocyte (26), it was hypothesized that CR3⁺ granulocytes might be the cells predominantly responsible for β -glucan-mediated tumoricidal activity.

In the current investigation, several mouse antitumor mAbs were examined in mouse syngeneic tumor models to determine whether their ability to mediate tumor regression could be significantly enhanced by the coadministration of i.v. soluble yeast β -glucan. Significant enhancement of tumor regression and survival was demonstrated

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with each therapeutic mAb when it was used in combination with β -glucan. The enhancement of tumoricidal activity mediated by β -glucan therapy was shown to be dependent upon CR3⁺ granulocytes that targeted tumors opsonized with iC3b.

MATERIALS AND METHODS

Antibodies and Other Reagents. The hybridoma producing 11C1 IgG2a anti-murine mammary tumor virus (MMTV; Ref. 30) was generously provided by Dr. Hiroshi Fugii (Department of Molecular Immunology, Roswell Park Cancer Institute, Buffalo, NY). Purified 14.G2a IgG2a anti-G_{D2} mAb (31, 32), as well as the hybridoma, was generously provided by Dr. Ralph A. Reisfeld (Research Institute of Scripps Clinic, La Jolla, CA). The BCP8 hybridoma producing IgG2b antihuman MUC1 mAb (33) was kindly provided by Dr. Ian F. C. McKenzie (Austin Research Institute, Heidelberg, Australia). The hybridoma producing the rat antimouse granulocyte mAb RB6-8C5 (Ly-6G; anti-Gr-1; Ref. 34) was kindly provided by Dr. Emil Unanue (Washington University School of Medicine, St. Louis, MO). The B5 hybridoma secreting mouse IgG2a mAb specific for the human high M_r melanoma antigen was obtained from the American Type Culture Collection (Manassas, VA), and the isolated IgG was used as a "nonspecific" mAb control for mouse tumor therapy protocols. Each hybridoma was adapted to grow in BD Hybridoma medium with 1–2% FCS and then grown in bioreactor flasks (BD Biosciences, San Jose, CA) to generate a spent medium rich in mAb that was subsequently purified using sequential steps of ammonium sulfate precipitation, Mono-Q FPLC chromatography, and Mono-S FPLC chromatography (26). Purified mAbs were sterilized by ultrafiltration, and any detectable lipopolysaccharide was removed by extraction with Triton X-114 (35).

Goat antimouse antibodies to IgM, IgG, and C3 labeled with FITC were purchased from ICN Biomedicals/Cappel (Aurora, CA) and used for analysis of immunoglobulin and C3 opsonization of tumor cell suspensions using flow cytometry (BD FACScan; BD Biosciences Immunocytometry Systems, San Jose, CA). Antimouse CD45-PerCP-Cy5.5, antimouse CD80-FITC, anti-CD11c-FITC, anti-Gr-1-PE, and appropriately labeled isotype controls were purchased from BD Biosciences Pharmingen. Rat antimouse F4/80-FITC and an isotype control were obtained from Caltag Laboratories (Burlingame, CA).

Therapeutic β -Glucans. A soluble β -glucan known as neutral soluble glucan (NSG) β -glucan that was estimated to be M_r ~10,000 in size was obtained from Biopolymer Engineering. NSG was derived from baker's yeast and came from material that had been generated several years ago by Alpha Beta Technology, Inc. (Worcester, MA) and is similar to the product that they had marketed as soluble yeast β -glucan through Molecular Probes, Inc. (Eugene, OR) approximately 7 years ago. The ability of this latter material to bind to and prime human and murine CR3 for toxicity of iC3b-opsonized tumor cells was described previously (10, 20, 36).

Mice and Tumor Models. Normal BALB/c and C57Bl/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) or National Cancer Institute (Frederick, MD). Heterozygous C3-deficient (C3^{+/-}) mice (21) were purchased from The Jackson Laboratory and used to establish a breeding colony from which were derived both homozygous deficient (C3^{-/-}) and their wild-type (C3^{+/+}) C57Bl/6 littermates. A breeding colony of C57Bl/6 CR3-deficient (CD11b^{-/-}) mice (22) and their wild-type (CD11b^{+/+}) C57Bl/6 littermates was obtained from Dr. Tanya Mayadas-Norton (Brigham & Women's Hospital and Harvard Medical School, Boston, MA). The phenotypes of the C3^{-/-} and CR3^{-/-} mice and their littermates were confirmed by assays for serum C3 using quantitative radial immunodiffusion and for blood neutrophil CD11b expression using immunofluorescence staining and flow cytometry analysis, respectively.

The BALB/c mammary carcinoma known as Ptas64 (or 64PT) was obtained from Dr. Wei-Zen Wei (Karmanos Cancer Center and Wayne State University, Detroit, MI). This tumor line expresses a MMTV membrane antigen detectable with the 11C1 mAb. Previous studies showed that normal BALB/c serum contained naturally occurring antibodies reactive with Ptas64 that opsonized the tumor cells growing *in vivo* with IgM, IgG, and C3, and that additional *i.v.* injections of 11C1 mAb produced increased surface uptake of IgG and C3 (26). Four groups of six to eight mice received *s.c.* injection of 0.5 to 1.0 × 10⁶ cells in a mammary fat pad, and a tumor was allowed to form over a 7–10-d period. When tumor diameters reached 3–4 mm as measured by calipers as an average

across the tumor length and width, therapy was initiated. The four groups included: (a) control group receiving *i.v.* PBS or *i.v.* nonspecific mAb; (b) 100 μ l of 11C1 mAb in saline (2 mg/ml) *i.v.* every 3rd day; (c) 100 μ l of β -glucan (NSG; 4 mg/ml in saline) *i.v.* daily; and (d) a combination of 11C1 mAb every 3rd day and daily injections of β -glucan. Tumor diameter was measured every 3rd day, and mice were sacrificed when tumor diameters reached 15 mm.

RMA-S is a C57Bl/6 lymphoma that expresses G_{D2} ganglioside but is defective in peptide loading of MHC class I. The RMA-S parent line and RMA-S cells transfected with human MUC1 were provided by Dr. Olivera J. Finn (Pittsburgh Cancer Institute, Pittsburgh, PA; Ref. 37). For use in a *s.c.* tumor model, 2–3 × 10⁶ RMA-S lymphoma transfected with human MUC1 (RMA-S-MUC1) cells were implanted *s.c.* in C57Bl/6 mice in or near a mammary fat pad. After 5–10 days when tumors of 4–6 mm appeared, therapy was initiated with either 14.G2a anti-G_{D2} or BCP8 anti-MUC1 mAb, with or without NSG β -glucan using four groups of mice: (a) 200 μ g of B5 nonspecific control mAb given *i.v.* every 3rd day; (b) 400 μ g of NSG β -glucan given *i.v.* daily; (c) 100 μ g of 14.G2a anti-G_{D2} mAb or 200 μ g of BCP8 anti-MUC1 mAb given *i.v.* every 3rd day; and (d) combined treatment with mAb and β -glucan. Therapy was continued for 2 or 3 weeks (as indicated), with tumor measurements made every 3rd day, and mice were sacrificed if tumors reached 15 mm in diameter. Mice were observed for tumor-free survival over a total period of 90–120 days.

A liver tumor model was carried out using the RMA-S lymphoma line in combination with 14.G2a mAb to G_{D2} ganglioside (100 μ g given *i.v.* every 3rd day). Mice were similarly divided into four groups that were treated beginning 5 days after *i.v.* injection of 3 × 10⁵ cells with: (a) *i.v.* PBS (control); (b) *i.v.* NSG β -glucan (400 μ g/day); (c) 14.G2a mAb; or (d) both NSG β -glucan and 14.G2a mAb. Therapy was continued for a period of 3 weeks, and the mice were then observed for long-term tumor-free survival.

Lewis lung carcinoma cells (LL/2, CRL-1642) originally derived from C57Bl/6 mice were obtained from the American Type Culture Collection and transfected with a plasmid containing cDNA for human MUC1 that was provided by Dr. Olivera Finn (37). A LL/2 line expressing a uniformly high surface density of MUC1 was selected by fluorescence-activated cell sorting of cells stained with BCP8-FITC mAb (MoFlo High Speed Cell Sorter; Dako-Cytomation, Fort Collins, CO). An additional selection was made by passaging the cell line two times in C57Bl/6 mice given the cells *s.c.* A tumor line was selected that expressed uniformly high levels of surface MUC1 and was capable of generating *s.c.* tumors in C57Bl/6 mice receiving injection of as few as 5 × 10⁵ cells. Therapy of mice bearing these *s.c.* tumors was initiated after 7 days when tumors were 1–2 mm in diameter. Four groups of six C3-deficient or their wild-type littermate C57Bl/6 mice were treated with: (a) *i.v.* PBS every 3rd day (control); (b) 400 μ g of NSG β -glucan given *i.v.* daily; (c) 200 μ g of BCP8 anti-MUC1 mAb given *i.v.* every 3rd day; and (d) combined therapy with BCP8 mAb and NSG β -glucan. Therapy was given for 3 weeks with measurement of tumor diameters every 3rd day, and mice were sacrificed when tumors reached 15 mm in diameter. Mice were observed over a total period of 90 days for tumor-free survival.

Analysis of Mice with Mammary Tumors for Leukocytosis. Two groups of six BALB/c mice were compared for peripheral blood leukocyte counts after mammary fat pad implantation of 1 × 10⁶ Ptas64 mammary adenocarcinoma cells in one of the groups of mice. After tumors appeared on day 8, peripheral blood was collected every other day for analysis. Absolute leukocyte counts were performed using BD Tru-Count tubes (BD Biosciences, San Jose, CA) according to the instructions from the manufacturer. In brief, 50 μ l of whole blood were stained in Tru-Count tubes containing a known number of beads with 1.0 μ l of PerCP-Cy5.5-conjugated rat antimouse CD45 mAb. After 20 min on ice, erythrocytes were lysed by adding 450 μ l of fluorescence-activated cell sorter lysing solution (BD Biosciences, San Jose, CA), and samples were immediately analyzed by BD FACScan. During data acquisition, the threshold was set on FL3, allowing analysis of beads and CD45⁺ cells only. Absolute leukocyte counts were calculated according to the formula:

Number of events in region containing leukocytes

Number of events in absolute count bead region

$$\times \frac{\text{Number of beads per test}}{\text{Test volume}} = \text{Absolute count of leukocytes}$$

Tumor Therapy with Granulocyte-Depleted Mice. The Ptas64 mammary tumor model in BALB/c mice was carried out as above with some therapy groups added in which the mice were depleted of granulocytes using a previously reported method that involves treatment with the rat antimouse granulocyte mAb RB6-8C5, also known as anti-Gr-1 (38). To prevent infections in the granulocyte-depleted mice, all therapy groups of the mice were maintained in laminar flow hoods, and tetracycline antibiotic was added to their drinking water (500 mg of tetracycline and 50 g sucrose/liter water). Because effective tumor regression requires serum complement to opsonize tumors with iC3b, pilot studies were carried out to determine conditions of chronic granulocyte depletion that would give time for repletion of serum complement function before initiating tumor therapy. Mice received i.p. injection of 300 μ g of RB6-8C5 mAb 3 days before beginning therapeutic treatment of the tumors with 11C1 mAb and NSG β -glucan. After the initial depletion of granulocytes, additional i.v. injections of 300 μ g of RB6-8C5 were given at 3-day intervals by mixing together the RB6-8C5 mAb with the 11C1 mAb so that both mAbs were given in a single i.v. injection. Tests of sera from mice treated with the RB6-8C5 showed that complement activity had returned to normal levels by day 3, whereas peripheral blood smears stained with Wright-Giemsa showed virtually no remaining neutrophils or eosinophils. Continued i.v. injections of RB6-8C5 did not affect serum complement levels significantly because granulocyte numbers were too low to require significant complement consumption for cytotoxicity. There was also no evidence for depletion of complement during the tumor therapy period by tests of sera from RB6-8C5-treated mice for their ability to opsonize 11C1-opsonized Ptas64 tumor cells with C3 *in vitro* as determined by staining with anti-C3-FITC and flow cytometry. To confirm that the RB6-8C5 mAb depleted only granulocytes that express high surface levels of Gr-1 and not monocytes, macrophages, and dendritic cells that express lower surface densities of Gr-1, additional tests were carried out on mice undergoing the RB6-8C5 depletion protocol to check for the presence and number of these other myeloid cell types in bone marrow, spleen, and peripheral blood. Monocytes were identified in blood samples using flow cytometry and double-staining with anti-Gr-1-PE and anti-CD80-FITC, macrophages were identified with anti-Gr-1-PE and F4/80-FITC, and dendritic cells were identified with anti-Gr-1-PE and anti-CD11c-FITC.

Graphing and Statistical Analysis of Data. All data from mouse tumor therapy protocols were entered into Prism 3.0 (Graph Pad Software, San Diego, CA) to generate graphs of tumor regression or survival. Student's *t* test was next used within Prism 3.0 to determine the significance of differences between data sets.

RESULTS

Combined Therapy with β -Glucan Significantly Enhances Tumor Regression Elicited with MAb. Previous research had shown that the therapeutic effect of β -glucans required naturally occurring antitumor antibodies that functioned to target tumor cells with iC3b (26). It was hypothesized that an additional infusion of an antitumor mAb could enhance β -glucan therapy. Two mAbs were examined with RMA-S-MUC1 implanted s.c. in C57Bl/6 mice (Fig. 1). The RMA-S-MUC1 cells express a high surface density of G_{D2} ganglioside but are resistant to CDC (not shown). Pilot studies with 14.G2a demonstrated maximal IgG and C3 uptake by s.c. tumors with a dose of 100 μ g of mAb given at 3-day intervals (not shown). Transfection of the RMA-S cells with human MUC1 allowed them to be alternatively targeted with BCP8 IgG2b anti-MUC1. A dose of 200 μ g of BCP8 given at 3-day intervals was shown to produce a maximal coating of IgG and C3 on s.c. tumors (not shown).

Similar to Ptas64 and its syngeneic BALB/c host (26), measurable levels of naturally occurring antibodies reactive with RMA-S were detected in the sera of its syngeneic C57Bl/6 host, and these functioned to opsonize RMA-S-MUC1 solid tumors *in vivo* with IgM, IgG, and C3. Thus, as expected, there was some tumor regression noted when mice were treated with i.v. β -glucan alone (Fig. 1). Likewise, each of the two mAbs had some ability to mediate tumor regression, with the 14.G2a being more effective, probably because of

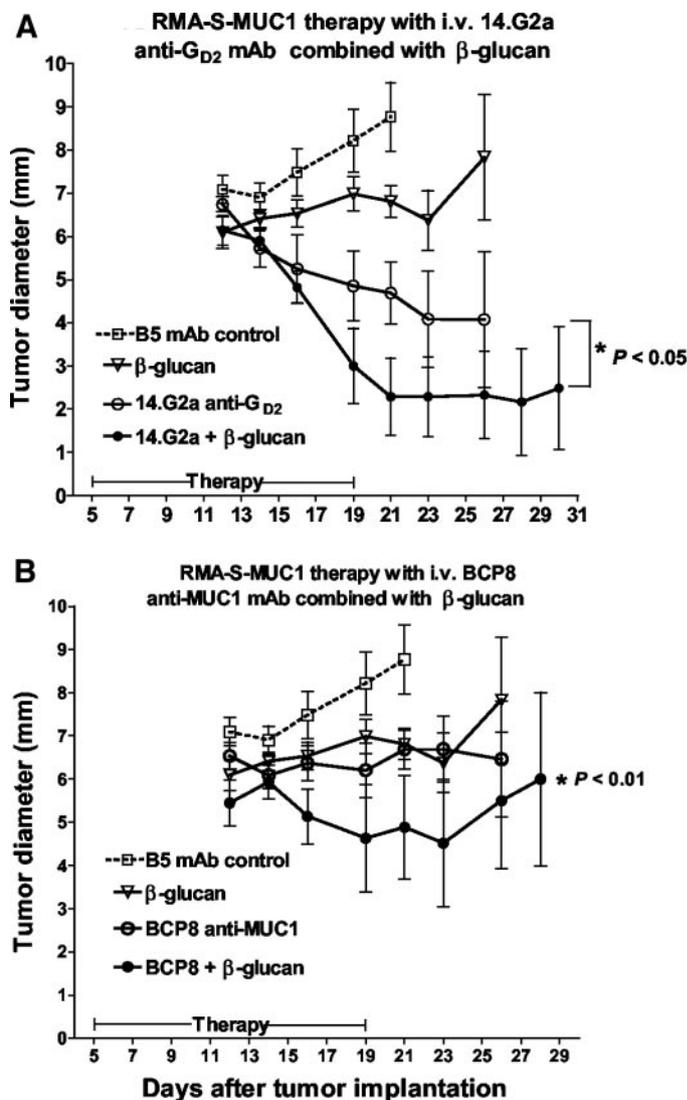


Fig. 1. Combined use of yeast β -glucan significantly enhances the regression of s.c. tumors produced by treatment with antitumor mAb alone. C57Bl/6 mice were implanted s.c. with RMA-S-MUC1 tumor cells and then 5 days later were treated with either 14.G2a anti-GD2 ganglioside (100 μ g every 3rd day) and/or NSG β -glucan (300 μ g/day) or BCP8 IgG2b anti-MUC1 (200 μ g every 3rd day) and/or NSG β -glucan (300 μ g/day). Immunotherapy was carried out for a total period of 14 days. Tumor measurements were taken over a 3-week period, and then survival was monitored (survival data shown in Fig. 2). With either mAb, the combined administration of NSG β -glucan resulted in significantly more tumor regression than with mAb therapy alone. Mean values and SE are shown.

the high G_{D2} antigen density of RMA-S-MUC1 cells. When the RMA-S-MUC1 cells were targeted with BCP8 anti-MUC1 instead of 14.G2a, the lower expression of MUC1 antigen was presumably responsible for a lower rate of tumor regression. Notably, the coadministration of β -glucan in both tumor models resulted in significantly more tumor regression than was observed with mAb therapy alone (Fig. 1). At the end of the 2-week therapy period, the mice were observed for tumor-free survival for a 4-month period (Fig. 2).

With the RMA-S-MUC1 tumors treated with 14.G2a anti-G_{D2} with or without β -glucan, 60% of the mice treated with mAb alone survived, whereas 80% of the mice survived that were treated with a combination of mAb plus β -glucan (difference not significant). The greater success of the therapy with this model is probably due to the high and stable expression of the G_{D2} antigen. When the same tumor cells were targeted with BCP8 anti-MUC1, there was no enhancement of survival with the mAb alone, but both the β -glucan and combined mAb plus β -glucan groups exhibited a comparable 20% survival.

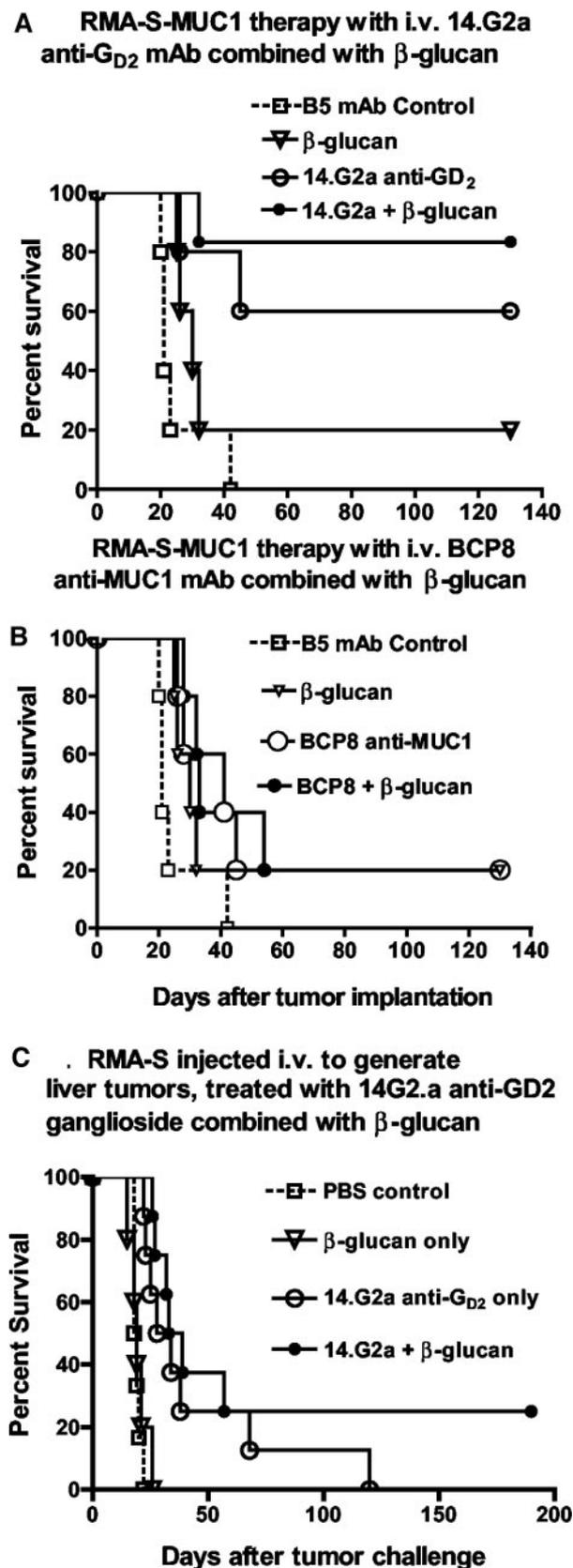


Fig. 2. Combined administration of soluble β -glucan enhances the survival of mice treated with antitumor mAbs. *A* and *B* represent the survival curves for the tumor therapy protocols described in the legend for Fig. 1. *C* demonstrates the enhanced survival of mice with RMA-S hepatic lymphomas treated with 14.G2a IgG2a anti-G_{D2} ganglioside in combination with β -glucan. As described in "Materials and Methods," mice received injections of RMA-S tumor cells i.v. to generate tumors in the liver, and after five days, the mice were given i.v. therapy with mAb and/or β -glucan. Therapy was administered for 3 weeks, and then the survival of mice was recorded.

Examination of the tumors removed from these mice showed that <25% of the tumor cells continued to express MUC1, suggesting that tumor escape had probably occurred because the tumor cells with MUC1 had been killed selectively by the therapy. Before implantation, the RMA-S-MUC1 cells had been sorted by fluorescence-activated cell sorter, and a line had been selected for tumor implantation that uniformly expressed MUC1.

Tumor-free survival was then examined in a liver tumor model incorporating the RMA-S lymphoma, the C57Bl/6 syngeneic host, and the 14.G2a anti-G_{D2} mAb (Fig. 2C). When injected i.v., RMA-S forms hepatic tumors. RMA-S tumor cells are defective in their ability to load peptides in MHC class I and therefore are resistant to recognition and killing by cytotoxic T lymphocytes. Therapy with β -glucan alone had no therapeutic benefit, whereas therapy with 14.G2a alone did extend survival. Nevertheless, combined therapy with 14.G2a and β -glucan not only extended survival, but also 25% of mice were long-term survivors.

β -Glucan Adjuvant Function Requires Leukocyte CR3 and Serum C3. Previous studies had shown that the ability of β -glucan to mediate tumor regression was absent in either BALB/c CR3-deficient or 129/J C3-deficient mice (26). The current study examined CR3- or C3-deficient C57Bl/6 mice with RMA-S-MUC1 or LL/2 transfected with human MUC1 (LL/2-MUC1) s.c. tumors, respectively. With RMA-S-MUC1 tumors in wild-type mice, regression mediated either by mAb alone or β -glucan alone was comparable, and this was enhanced when mAb was combined with β -glucan (Fig. 3). Although the enhancement of mAb-mediated regression elicited by β -glucan was not significant, the combination of mAb plus β -glucan did produce significantly enhanced survival, with 40% of the mice remaining tumor free (Fig. 4). In comparison, there were no survivors in the groups treated with mAb alone or β -glucan alone. Finally, in the CR3-deficient mice, there was no regression with β -glucan alone, and although there was regression with mAb alone, there was no enhancement of mAb regression by combination with β -glucan, and none of the therapy groups exhibited long-term survival.

Another tumor was tested in wild-type *versus* C3-deficient C57Bl/6 mice. LL/2-MUC1 was implanted s.c. and treated with BCP8 anti-MUC1 mAb with or without β -glucan. In wild-type mice, there was no tumor regression after treatment with the BCP8 mAb alone, and therefore the regression elicited by combining the BCP8 with β -glucan was significant ($P < 0.05$; Fig. 5). A low level of naturally occurring antibody against the LL/2 tumor probably explained the regression observed in the groups receiving β -glucan alone. Likewise, therapy with β -glucan alone or mAb plus β -glucan exhibited a survival advantage compared with mAb alone (Fig. 6). By contrast, neither mAb nor β -glucan, separately or combined elicited regression (Fig. 5) or enhanced survival (Fig. 6) in C3-deficient mice.

β -Glucan-Mediated Tumor Regression Is Granulocyte Dependent. Previous *in vitro* studies had demonstrated that human and mouse monocyte/macrophages, neutrophils, and NK cells could each carry out β -glucan-mediated CR3-dependent cellular cytotoxicity against iC3b-opsonized tumor cells (10, 11, 20). However, attempts to identify the specific effector cell(s) required for this response in mouse tumor models had been unsuccessful (26). One mechanism that was likely to be involved in the recruitment of leukocytes was complement activation at the tumor site mediated by antitumor antibody, and this was recognized to occur in most tumor models via naturally occurring antibody, even in PBS control group tumors. Indeed, it was shown that there was a significant increase in peripheral blood granulocyte counts (leukocytosis) in mice with Ptas64 mammary tumors that was independent of mAb and/or β -glucan therapy (Fig. 7).

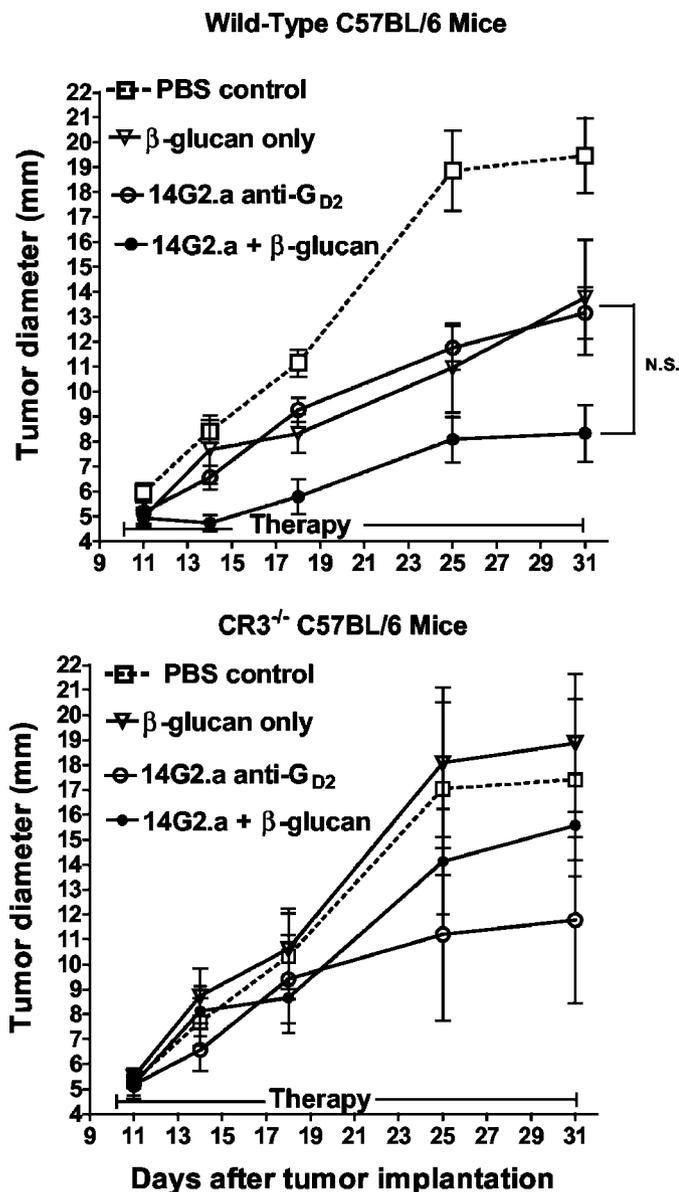


Fig. 3. The enhancement of tumor regression mediated by NSG β-glucan requires leukocyte CR3 and fails in CR3-deficient (CD11b^{-/-}) mice. Comparable groups of CR3-deficient C57BL/6 mice and their wild-type littermates were implanted s.c. with RMA-S-MUC1 tumor cells (2×10^6), and tumors of 5–6 mm in diameter were allowed to form over 10 days before carrying out immunotherapy for a total period of 21 days. Tumor measurements were made at the indicated times (Fig. 3), and then survival was monitored (Fig. 4). Mice received 14.G2a anti-G_{D2} ganglioside mAb (100 μg every 3rd day) and/or NSG β-glucan (400 μg daily). There was no difference in the rate of tumor regression produced by mAb therapy alone in the CR3-deficient versus wild-type mice, and there was no β-glucan enhancement of mAb-mediated tumor regression in the CR3-deficient mice. Even though the enhancement of mAb-mediated regression induced by β-glucan was not significant (n.s.), β-glucan did significantly enhance mAb-mediated survival (Fig. 4). Mean values ± SE of the mean are shown.

Complement activation releases C3a and C5a that function to recruit eosinophils, mast cells (C3a), neutrophils, and macrophages (C5a). Thus, recruitment might be equivalent in therapy versus PBS control group tumors, but only in tumors from mice receiving β-glucan would there be leukocytes with primed CR3 able to kill iC3b-opsonized tumor cells.

Because the major leukocyte type identified in Ptas64 tumors (with or without therapy) was the granulocyte (26), it was hypothesized that β-glucan-primed granulocytes might be predominantly responsible for tumoricidal activity. Treatment of mice with anti-Gr-1 mAb has been previously reported to deplete granulocytes (neutrophils plus

eosinophils) selectively with little or no effect on other leukocyte types (38). However, the Gr-1 antigen is known to be expressed at lower levels on all myeloid cell populations including monocyte/macrophages and dendritic cells. Thus, it was important to show that the protocol used in this study was selective for granulocytes and did not deplete monocytes, macrophages, or dendritic cells. Evaluation of blood and splenic leukocytes by flow cytometry confirmed nearly complete depletion of peripheral granulocytes (Fig. 8). However, anti-Gr-1 treatment had little effect on Gr-1^{high} granulocytes in the bone marrow. Presumably these cells are killed as soon as they exit from the marrow. Examination of peripheral blood by flow cytometry showed no depletion of Gr-1^{low}CD80⁺ monocytes, whereas virtually all Gr-1^{high}CD80⁻ neutrophils were missing (Fig. 8). Tests of splenocytes showed no detectable depletion of Gr-1^{low}F4/80⁺ macrophages (Fig. 8) or Gr-1^{low}CD11c⁺ dendritic cells (not shown). There was also no evidence for macrophage or dendritic cell depletion from bone marrow (not shown).

To determine the role of granulocytes in this form of tumor immunotherapy, mAb and β-glucan therapy of BALB/c mice with Ptas64 mammary tumors was carried out in the same way as in the other protocols, but some mice were granulocyte-depleted using anti-Gr-1 mAb (Fig. 9). Ptas64, a mammary carcinoma syngeneic to BALB/c mice, is latently infected with MMTV and expresses a membrane surface MMTV tumor antigen detectable with the 11C1 IgG2a mAb. Pilot studies demonstrated that a 200 μg i.v. dose of the 11C1 given at 3-day intervals produced a maximal coating of IgG and C3 on

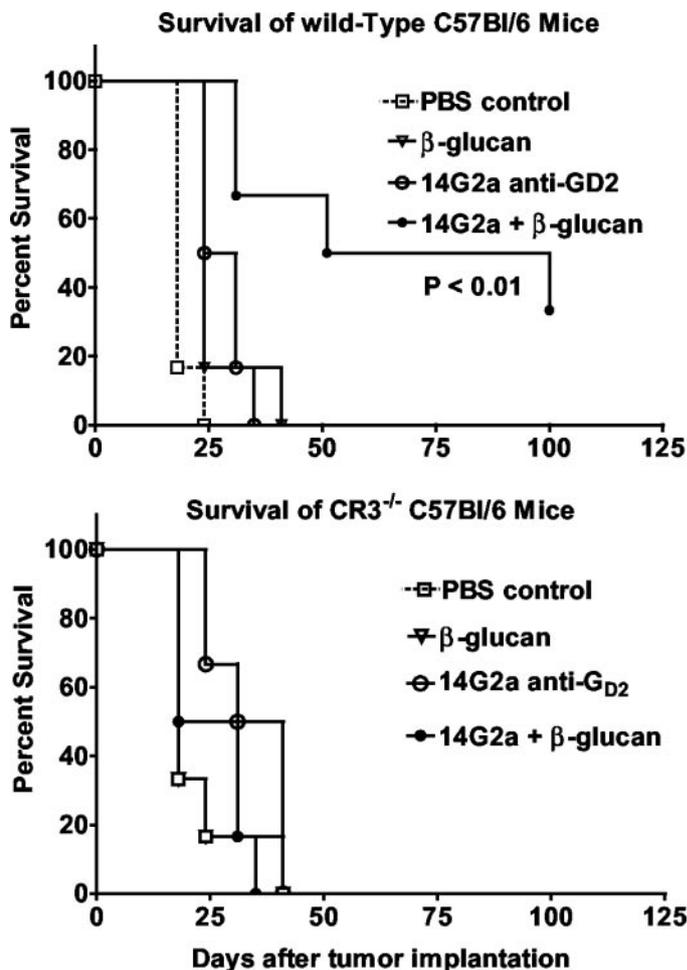


Fig. 4. NSG β-glucan combined with antitumor mAb therapy enhances survival of wild-type but not CR3-deficient mice. These data represent the survival curves for the protocol described in Fig. 3.

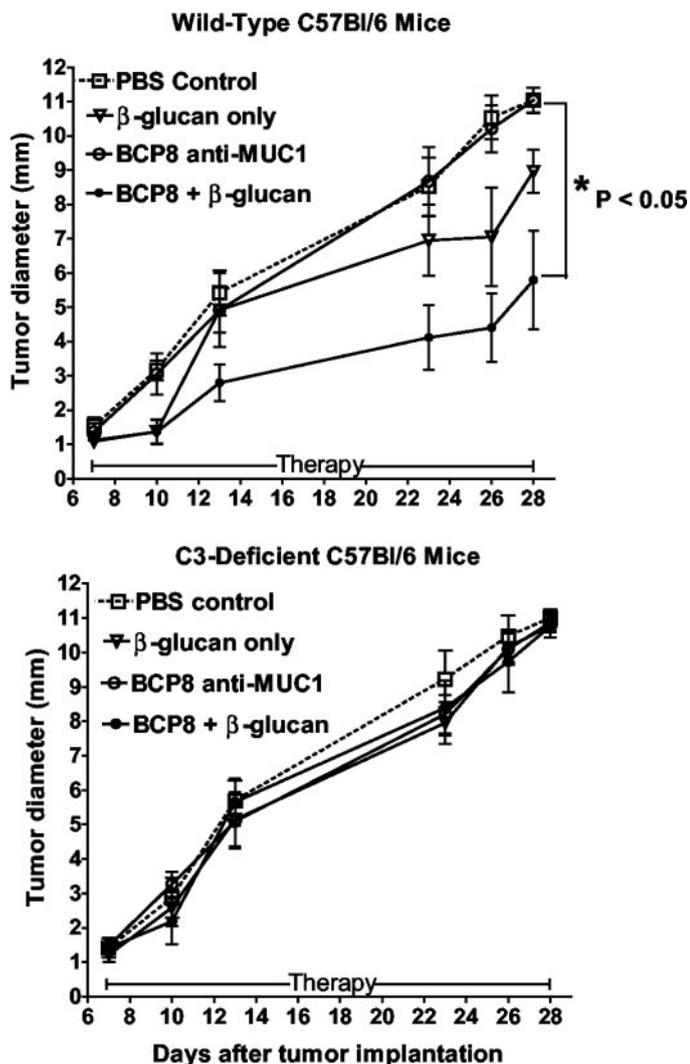


Fig. 5. The enhanced tumor regression mediated by NSG β-glucan when combined with antitumor mAb requires plasma C3 and does not occur in C3-deficient mice. C3-deficient (C3^{-/-}) mice and their wild-type littermates on a C57BL/6 background were implanted s.c. with 1×10^6 LL/2-MUC1. After allowing 7 days for development of small tumors, mice were treated with BCP8 IgG2b anti-MUC1 mAb (200 μg every 3rd day) and/or NSG β-glucan (400 μg daily) for a total therapy period of 3 weeks. Therapy with BCP8 mAb alone did not produce significant tumor regression in either wild-type or C3-deficient mice, whereas the combination of BCP8 mAb and soluble β-glucan produced significant tumor regression in wild-type but not in C3-deficient mice. Mean values ± SE of the mean are shown.

individual mammary tumor cells after surgical removal of tumors and flow cytometry analysis (not shown). To prevent any complement depletion that might occur during the peak of complement-dependent granulocyte killing with the anti-Gr-1 mAb, mice were treated with the anti-Gr-1 i.p. three days before beginning mAb plus β-glucan therapy and then the mice were given additional i.v. injections of the anti-Gr-1 mAb at the same time as they were given the i.v. 11C1 therapeutic mAb. Serum complement levels were monitored at the beginning and during mAb plus β-glucan therapy by examining the ability of small serum samples from the mice to opsonize 11C1-opsonized Ptas64 cells *in vitro* with C3 as determined by staining with antimouse C3-FITC and flow cytometry. No evidence for complement depletion via the anti-Gr-1 treatment was obtained (not shown). Not only was the tumor regression mediated by mAb plus β-glucan completely abrogated by granulocyte depletion, but also the tumors in the granulocyte-depleted mice grew at a significantly faster rate than

the tumors in the PBS control group during the last week of tumor measurements (Fig. 9). Thus, granulocytes (neutrophils and/or eosinophils) play an essential role in mediating tumor regression in therapy mediated by mAb plus β-glucan.

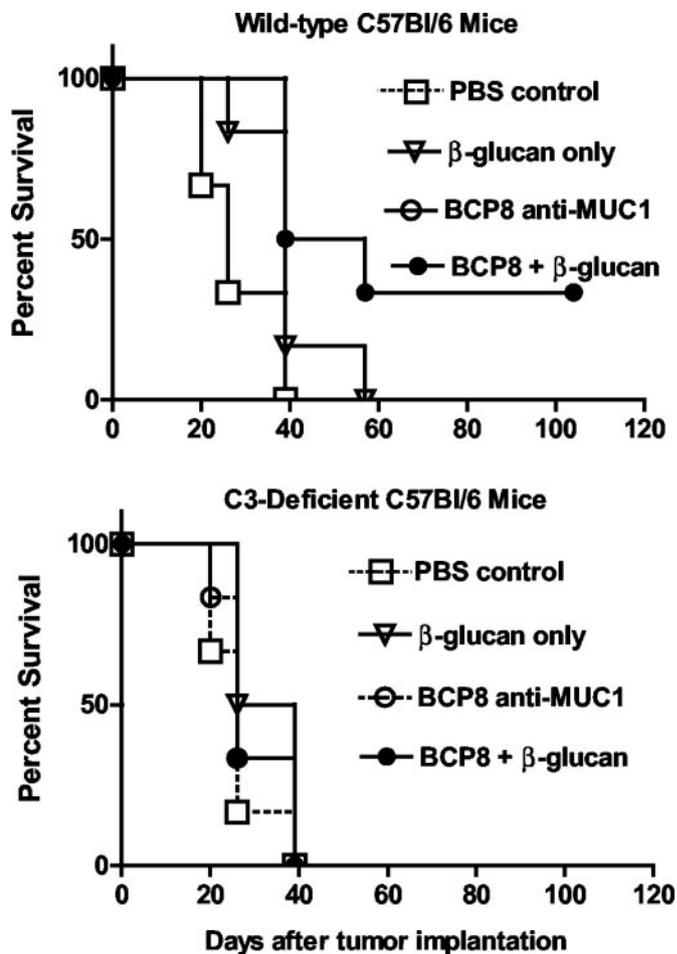


Fig. 6. The enhanced survival induced by the combination of soluble β-glucan with antitumor mAb requires C3 and does not occur in C3-deficient mice. These data represent the survival curves for the tumor therapy protocol described in Fig. 5.

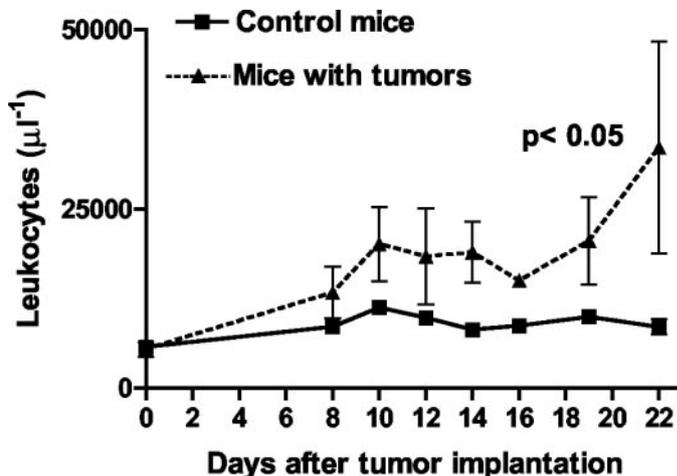


Fig. 7. Mammary tumor development elicits significant leukocytosis. Peripheral blood was collected from either BALB/c control mice or mice implanted in a mammary fat pad with 1×10^6 Ptas64. Absolute leukocyte counts were performed by flow cytometry as described in “Materials and Methods.” Palpable tumors were detected 8 days after implantation. After the 8th day, a significant leukocytosis was observed in tumor-bearing mice, whereas control mice had normal leukocyte counts. Mean values ± SE of the mean are shown.

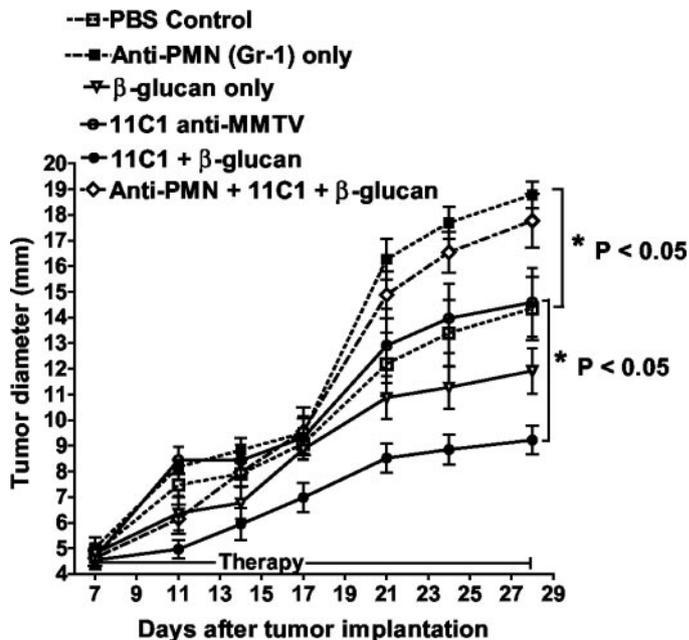
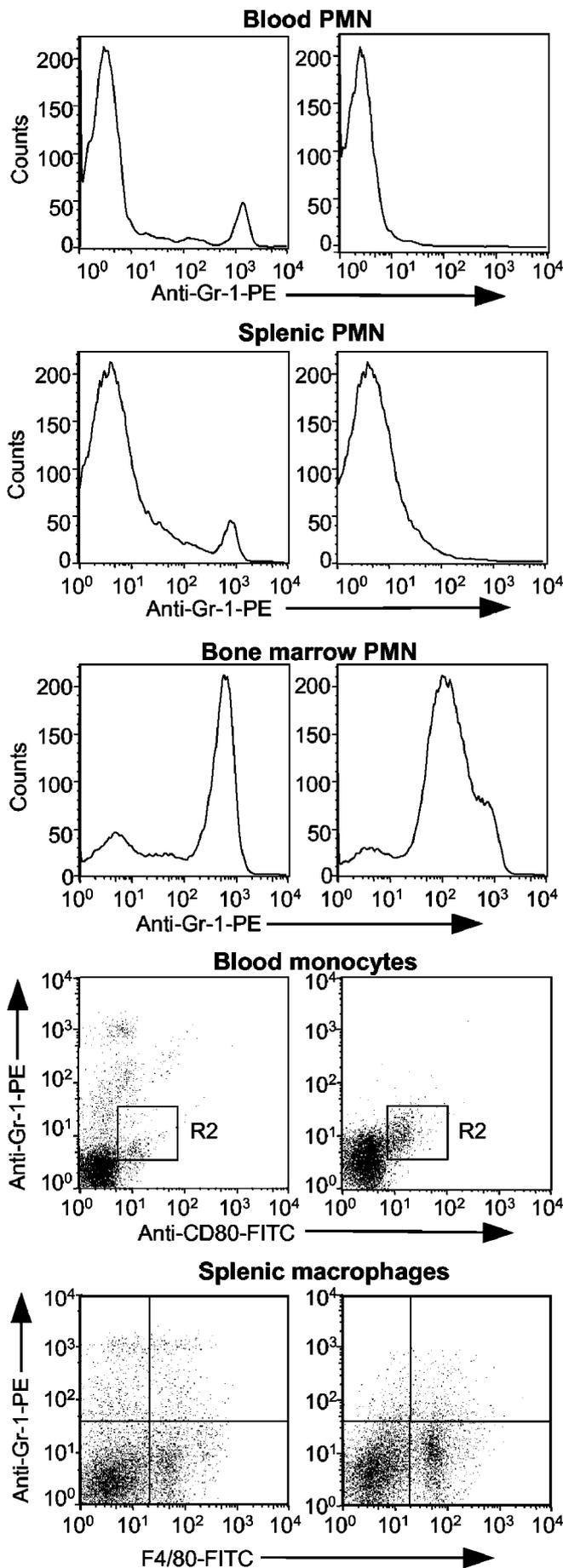


Fig. 9. The tumoricidal activity of immunotherapy with NSG β-glucan requires granulocytes and fails in mice depleted of granulocytes with anti-Gr-1 mAb. BALB/c mice were implanted with Ptas64 mammary carcinoma cells in a mammary fat pad, and a tumor was allowed to form over 7 days before initiating immunotherapy. Mice were treated with 11C1 IgG2a anti-MMTV mAb and/or NSG β-glucan (400 μg daily) for a total period of 3 weeks. Granulocytes were depleted from some of the groups of mice as described in "Materials and Methods." The combined use of soluble β-glucan produced significantly greater tumor regression than did treatment with 11C1 mAb only ($P < 0.05$, lower bracket and *). Mean values ± SE of the mean are shown.

DISCUSSION

This investigation showed that both the tumor regression and increased survival mediated by monoclonal antitumor antibodies could be significantly enhanced if they were given in combination with β-glucan. The ability of β-glucan to enhance the activity of antitumor mAbs required that the mAbs activate complement and deposit iC3b on tumor cells for recognition by CR3⁺ granulocytes.

Previous reports had shown that β-glucans functioned as a monotherapy through naturally occurring antitumor antibodies that deposited iC3b on tumor cells, and that therapy was less effective in young mice that had lower levels of such natural antitumor antibodies (26). Therapy failed in mice with inherited severe combined immunodeficiency (that have no B and T lymphocytes and cannot make natural antibodies) but could be reconstituted by i.v. injection of natural antibodies isolated from normal mouse sera. However, even in adult wild-type mice, tumor escape occurred when tumors lost the antigens recognized by naturally occurring antibodies such that they were no longer targeted with iC3b (26).

Fig. 8. Treatment of mice with i.v. RB6-8C5 anti-Gr-1 mAb selectively depletes granulocytes but not monocytes, macrophages, or dendritic cells. As described in "Materials and Methods," mice received injections of anti-Gr-1 mAb, first by i.p. injection and then 3 days later by i.v. injections repeated at 3-day intervals. Flow cytometry was used to assess the presence of granulocytes (PMN) in blood, spleen, and bone marrow in normal (left histograms) versus anti-Gr-1 treated mice (right histograms). The top two histograms show that anti-Gr-1 treatment effectively removed Gr-1⁺ PMN from the blood and spleen but had virtually no effect on Gr-1⁺ PMN in the bone marrow. Monocytes in region 2 (boxed area marked R2) showed no depletion when untreated control mice (left histogram) were compared with the anti-Gr-1-treated mice (right histogram). Similar studies carried out with bone marrow macrophages and with splenic and bone marrow dendritic cells likewise provided no evidence for depletion in the anti-Gr-1-treated mice (not shown). For the analysis of each cell population, staining was first carried out with anti-CD45-PerCP-Cy5.5, and a gate for analysis was established that included all CD45⁺ leukocytes.

It was hypothesized that the β -glucan-mediated immunotherapy could be enhanced by the coadministration of antitumor mAbs specific for a highly expressed and stable tumor antigen. The requirement that such mAbs activate complement was confirmed in experiments that demonstrated a failure of β -glucan to enhance mAb-mediated tumor regression or survival in C3-deficient mice. Others have also shown a lack of β -glucan enhancement of antitumor mAbs that did not activate complement (39). Thus, β -glucan cannot enhance the therapeutic activity of humanized mAbs that have been engineered in such a way that they do not activate complement. The majority of humanized mAbs containing the human IgG1 Fc-region have been shown to activate complement, such as Herceptin, Rituxan, and Erbitux (8, 40–43). With the exception of Rituxan, CDC does not represent a significant mechanism of tumoricidal activity and β -glucan does not alter the efficiency of CDC. Instead, β -glucan primes the CR3 of granulocytes to trigger the killing of tumor cells that have been targeted by mAb-mediated complement activation with surface-bound iC3b.

The RMA-S lymphoma was chosen for tumor therapy because it exhibits a high surface expression of G_{D2} ganglioside tumor antigen and forms liver tumors after i.v. challenge. RMA-S is also resistant to anti-G_{D2}-mAb-mediated CDC but sensitive to antibody-dependent cell-mediated cytotoxicity. Normal C57Bl/6 mouse sera contain naturally occurring antibodies to RMA-S that partially opsonize the tumor with IgG and C3 *in vivo*, but RMA-S is defective in peptide loading of MHC class I molecules, thus preventing recognition by CD8⁺ cytotoxic T cells. An i.v. challenge with RMA-S produced liver tumors that survived 2 weeks of mAb therapy, even when therapy was initiated 5 days after tumor challenge. Although monotherapy with mAb did enhance survival, only the combined therapy with β -glucan produced long-term tumor-free survival.

Combining β -glucan with antitumor mAb elicited a significant enhancement of tumor regression in four additional syngeneic tumor models in either BALB/c or C57Bl/6 mice. The success of therapy in generating long-term survival appeared to depend on tumor antigen density and stability. Tumor escape was characterized by a loss of tumor antigen, resulting in tumor cells lacking the bound iC3b required for recognition by the β -glucan-primed CR3 of recruited granulocytes. With the RMA-S-MUC1 tumor model, there was 80% long-term survival when the G_{D2} tumor antigen was targeted with 14.G2a mAb, but only 20% survival when MUC1 was targeted with BCP8 mAb. Examination of tumors that escaped the combined BCP8 and β -glucan therapy showed that <25% of tumor cells continued to express MUC1 and bear membrane iC3b. This should not be a problem in targeting MUC1 on human tumors, because MUC1 is usually overexpressed and stable. It is particularly of interest to note that BCP8 anti-MUC1 mAb monotherapy had no effect on either tumor growth rate or survival from a challenge with MUC1-transfected LL/2, and yet when BCP8 was used in combination with β -glucan, it elicited significant tumor regression and long-term survival (Figs. 5 and 6).

Previous reports that had studied tumor regression mediated by β -glucan without simultaneous mAb therapy had shown a requirement for both CR3 in a BALB/c tumor model and serum C3 in a 129/J tumor model (26). The current investigation confirmed a similar role for CR3 and C3 in β -glucan enhanced mAb therapy in C57Bl/6 mice that were examined for both tumor regression and long-term tumor-free survival.

In addition to CR3, others have recently reported the existence of a distinct type of macrophage β -glucan receptor known as dectin-1 (44). Dectin-1 was shown to be highly expressed on thioglycolate-elicited peritoneal macrophages, whereas much smaller amounts of dectin-1 were observed on resident peritoneal macrophages or granulocytes,

and none was detectable on NK cells (45). The current investigation does not exclude a function of dectin-1 but shows an absolute requirement for granulocyte CR3. Wild-type, but not CR3-deficient granulocytes, have been shown to bind NSG β -glucan, indicating that CR3 is the major receptor for soluble single-chain β -glucan on mouse granulocytes (20). CR3 is not only required for recognition of soluble β -glucan by granulocytes, but also is needed for triggering cytotoxicity of tumors coated with the CR3 target ligand iC3b.

In vitro experiments have previously shown that soluble β -glucan was able to prime the CR3 of macrophages, neutrophils, and NK cells for cytotoxicity of iC3b-coated tumor cells (10, 11, 20). The current investigation indicated that granulocytes were primarily responsible for β -glucan-mediated tumor regression *in vivo*. Granulocytes are recruited by tumors independently of mAb and β -glucan therapy, perhaps because of natural antibody activation of complement within tumors that releases the potent chemotactic factor C5a. Flow cytometry analysis of tumor cell suspensions showed that Gr-1⁺ granulocytes were the major CR3⁺ leukocyte type within tumors, and depletion of granulocytes with anti-Gr-1 confirmed the major role of granulocytes in β -glucan-mediated tumor regression.

An unexpected finding was that granulocytes appeared to play a role in tumor regression that was independent of mAb and β -glucan therapy. Tumors in granulocyte-depleted mice grew significantly faster than tumors in untreated control mice. Considering the leukocytosis that occurs in untreated mice with tumors, it appears possible that recruited granulocytes have some ability to kill tumor cells opsonized with natural antibody and iC3b, perhaps through C3-receptor enhanced antibody-dependent cell-mediated cytotoxicity because some of the natural antitumor antibody is IgG (26).

In conclusion, this investigation showed that the therapeutic efficacy of antitumor mAbs could be enhanced significantly by simultaneous administration of β -glucan. Furthermore, preliminary data have shown a similar enhancement of tumor regression when β -glucan is given in combination with tumor vaccines that generate antitumor antibodies.⁴ β -Glucan functions by recruiting granulocytes as tumor killer cells that are triggered via CR3 recognition of tumor cell-bound iC3b. This is a novel effector mechanism for antitumor mAb therapy that is additive to all other mechanisms of mAb-mediated tumor regression. As demonstrated with BCP8 anti-MUC1 mAb therapy of LL/2-MUC1, β -glucan can elicit significant regression and long-term survival, even with a mAb that has virtually no therapeutic effect on tumors when used as monotherapy. It is proposed that the previously reported inconsistent activity of β -glucan in cancer patients was likely due to the variable presence of natural or elicited antitumor antibodies. This investigation demonstrates that β -glucans can generate a more consistent tumoricidal response if combined with an antitumor mAb. Considering the low incidence of side effects associated with β -glucans, strong consideration should be given to the inclusion of β -glucan in mAb tumor therapy.

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⁴ G. D. Ross, unpublished data.

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