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Yeast β -Glucan Amplifies Phagocyte Killing of iC3b-Opsonized Tumor Cells via Complement Receptor 3-Syk-Phosphatidylinositol 3-Kinase Pathway¹

Bing Li², Daniel J. Allendorf², Richard Hansen, Jose Marroquin, Chuanlin Ding, Daniel E. Cramer and Jun Yan³

Tumor Immunobiology Program, James Graham Brown Cancer Center, University of Louisville, Louisville, KY 40202

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▶ Abstract

Anti-tumor mAbs hold promise for cancer therapy, but are relatively inefficient. Therefore, there is a need for agents that might amplify the effectiveness of these mAbs. One such agent is β -glucan, a polysaccharide produced by fungi, yeast, and grains, but not mammalian cells. β -Glucans are bound by C receptor 3 (CR3) and, in concert with target-associated complement fragment iC3b, elicit phagocytosis and killing of yeast. β -Glucans may also promote killing of iC3b-opsonized tumor cells engendered by administration of anti-tumor mAbs. In this study, we report that tumor-bearing mice treated with a combination of β -glucan and an anti-tumor mAb show almost complete cessation of tumor growth. This activity evidently derives from a 25-kDa fragment of β -glucan released by macrophage processing of the parent polysaccharide. This fragment, but not parent β -glucan, binds to neutrophil CR3, induces CBRM 1/5 neopeptide expression, and elicits CR3-dependent cytotoxicity. These events require phosphorylation of the tyrosine kinase, Syk, and consequent PI3K activation because β -glucan-mediated CR3-dependent cytotoxicity is greatly decreased by inhibition of these signaling molecules. Thus, β -glucan enhances tumor killing through a cascade of events, including in vivo macrophage cleavage of the polysaccharide, dual CR3 ligation, and CR3-Syk-PI3K signaling. These results are important inasmuch as β -

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glucan, an agent without evident toxicity, may be used to amplify tumor cell killing and may open new opportunities in the immunotherapy of cancer.

▶ Introduction

Anti-tumor mAbs represent one of the earliest targeted therapies in clinical cancer care (1). At present, eight anti-tumor mAbs with anticancer indications have been approved by the Food and Drug Administration. The effector mechanisms mediated by these anti-tumor mAbs are diverse and include inhibition of growth factor activity, facilitation of Ab-dependent cell-mediated cytotoxicity and/or complement-dependent cytotoxicity, and the creation of immunoconjugates with toxins or radioisotopes (2). However, Ab therapy is not uniformly effective, even in patients whose tumors express a high level of tumor Ag. Developing novel strategies to maximize the efficacy of anti-tumor mAbs is necessary to overcome this limitation in cancer therapy.

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β -Glucans are biological response modifiers and have been used for centuries in traditional Asian medicine, in some cases for the treatment of malignancies (with varying and unpredictable success) (3, 4, 5). They are glucose polymers produced by a variety of plants and microorganisms, including oat, barley, mushroom, seaweed, some bacteria and blue-green algae, and yeast (6). Yeast β -glucans are long polymers of $\beta(1, 3)$ glucose, with 3–6% of the backbone glucose units possessing a $\beta(1, 6)$ branch. Their unique structure allows these polymers to form triple helices in solution, which have the ability to self-associate into large aggregates (7). Given the different preparation procedures, three formulations of yeast β -glucan, including whole glucan particles (WGP),⁴ soluble i.v. administrated β -glucan PGG with an average molecular mass of 150 kDa, and very small molecular mass (<20-kDa) β -glucan known as neutral soluble glucan (NSG) are generated and extensively studied in research and clinical treatment (8, 9, 10, 11, 12, 13). Earlier in vitro studies have demonstrated that soluble NSG yeast β -glucan bound to a lectin domain within the COOH-terminal region of the CD11b subunit of C receptor 3 (CR3; CD11b/CD18, $\alpha_m\beta_2$ integrin, Mac-1) (14). β -Glucans prime neutrophils, macrophages ($M\phi$), and NK cells for cytotoxicity against tumors opsonized with iC3b as a result of complement activation by anti-tumor mAbs or natural Abs. Although NSG has a rapid clearance rate (15), subsequent in vivo studies showed clear antitumor effects of combined administration of either NSG or WGP β -glucan daily and anti-tumor mAbs in murine models, effects that were dependent on serum C3 and granulocyte CR3 (10, 11, 12, 16, 17, 18). Thus, β -glucan-mediated tumor immunotherapy uses a novel mechanism by which innate immune effector cells are primed to kill iC3b-opsonized tumor cells. Despite this earlier work, the mechanisms involved in the in vivo priming of CR3 by β -glucan PGG and the signaling events that activate effector cells remain to be elucidated.

We show in this study that β -glucan PGG is processed by $M\phi$ to release a 25-kDa active moiety that subsequently primes neutrophil CR3 for biological activity. The dual occupancy of CR3 mediated by

inserted domain (I domain) ligand iC3b and lectin-like domain ligand β -glucan leads to cytotoxic responses to tumor cells. This process is dependent on the CR3-Syk-PI3K pathway. Thus, we provide a more complete understanding of the mechanism of action of β -glucan PGG for tumor therapy.

► **Materials and Methods**

In vivo tumor therapy

The murine tumor therapy protocols were performed in compliance with all guidelines and were approved by the Institutional Animal Care and Use Committee of the University of Louisville. To compare the therapeutic efficacy of large molecular mass β -glucan PGG vs low molecular mass NSG β -glucan, 1×10^6 RMA-S lymphoma cells that express GD2 ganglioside were implanted s.c. in a mammary fat pad of wild-type (WT) C57BL/6 mice. After 9 days, when the tumors reached palpable, groups of mice ($n = 5$) were treated with PBS or 14 G2a anti-GD2 mAb (100 μ g) with or without β -glucan PGG (200 or 400 μ g) or NSG β -glucan (200 or 400 μ g). The 14 G2a mAb was given i.v. every third day, whereas β -glucan was given i.v. daily. Therapy was continued for 3 wk. The 14G2a hybridoma was provided by R. Reisfeld (Research Institute of Scripps Clinic, La Jolla, CA). In another tumor therapy protocol, 1×10^6 RMA-S-MUC1 cells were implanted s.c. in a mammary fat pad of WT C57BL/6 or CR3^{-/-} mice ($n = 10$). After 7 days, when tumors of 1–2 mm appeared, therapy was initiated with BCP8 anti-MUC1 mAb (200 μ g i.v. twice weekly) with or without i.v. β -glucan PGG (1200 μ g/mouse, twice per week). The BCP8 hybridoma producing IgG2b anti-MUC1 mAb was provided by I. McKenzie (Austin Research Institute, Heidelberg, Australia). Therapy was continued for 3 wk, during which time tumor measurements by calipers were calculated as the average of perpendicular diameters twice weekly. Mice were sacrificed when tumors reached 12 mm in diameter, recommended by Institutional Animal Care and Use Committee guidelines. In animals treated with anti-MUC1 mAb + β -glucan, survival was monitored for a period of 100 days beyond the tumor implantation. Animals in the other groups had to be sacrificed with tumors of >12 mm diameter after 30–50 days.

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In vivo trafficking of soluble β -glucan PGG

The soluble β -glucan PGG (Biothera) was labeled with fluorescein dichlorotriazine (DTAF; Molecular Probes) suggested by the manufacturer. Groups of five C57BL/6 WT mice or CR3^{-/-} mice were given 1200 μ g of the DTAF β -glucan PGG by tail vein injection. The spleen and bone marrow from different groups of mice were collected at days 1, 3, and 7. Confocal microscopy and flow cytometry were performed to determine the phenotype of fluorescence-positive cells.

Isolation of the active moiety of soluble β -glucans

Murine resident peritoneal M ϕ were maintained in a bioreactor flask (Integra Biosciences) in M ϕ growth

serum-free medium (Invitrogen Life Technologies). DTAF-labeled β -glucan PGG was added into culture. Following 3 wk of cell culture, the cell-free fluid from the lower chamber of the bioreactor was collected. This material was separated by HPLC (Waters 1525; Waters) using a monophasic gradient and separated on a Sephacryl S-200 (GE Healthcare) column. DTAF-labeled dextran standards of known molecular masses established an elution molecular mass profile. Fractions containing DTAF-labeled material as detected by A_{490} on a Waters 2996 Photodiode Array Detector indicated a dominant peak containing material of 25 kDa and a smaller peak containing residual parent 150-kDa β -glucan PGG. The 25-kDa fragments were further purified and concentrated by ultracentrifugation with a Centriprep (Millipore) with 50- and 10-kDa cutoff membranes. These fractions were confirmed to contain hexose by the phenol-sulfuric acid method.

Direct binding of 25-kDa β -glucan fragments and intact β -glucan to neutrophils

Neutrophils were collected from either mice or humans and resuspended in complete RPMI 1640 that had been supplemented with 10 μ g/ml polymyxin B (Sigma-Aldrich) to neutralize adventitious LPS. Neutrophils and β -glucan were incubated at 37°C for 3 h. Murine neutrophils were incubated with 10 μ g/ml Fc block (rat anti-mouse CD16/32 mAb; BD Pharmingen), and human neutrophils were incubated with a 1/20 dilution of heat-inactivated human serum for 20 min at room temperature. When data were acquired by flow cytometry, the cells were gated by light scatter and propidium iodide exclusion was used on a control aliquot of cells to confirm $\geq 90\%$ cell viability.

Induction of CBRM 1/5 neoepitope on human neutrophils

One million neutrophils were added to the wells of 96-well plates and mixed with either the DTAF-labeled parent β -glucan PGG or the DTAF-labeled 25-kDa β -glucan active moiety or LPS. Neutrophils and β -glucan or LPS were incubated at 37°C in a 5% CO₂ humidified incubator for 3 h. The neutrophils were next stained with an optimized dilution of CBRM 1/5 mouse anti-human CD11b mAb that detects the activation neoepitope of CD11b.

In vitro CR3-dependent cellular cytotoxicity

In vitro cytotoxicity of SKOV-3 cells by β -glucan-primed human neutrophils or monocytes was analyzed using a real-time measure of the impedance of electrical current by viable target cells adhered to a conductor on the bottom of wells in a 16-well plate (Acea Biosciences), according to manufacturer's instruction (19). Briefly, 5 x 10³ SKOV-3 cells were placed into the wells of the Acea 16-well plates for 24 h. Following this, fresh human serum and sufficient trastuzumab were added to the adherent SKOV-3 cells. The cells were incubated for 30 min at 37°C to permit complement activation and deposition of human iC3b. Human neutrophils or monocytes were added to achieve E:T cell ratios of 20:1 with or without parent β -glucan PGG or the 25-kDa fragments. Cells were incubated at 37°C in a humidified 5% CO₂ incubator for 12 h. Cytotoxicity was calculated by measuring the relative decrease in current impedance among wells containing iC3b-opsonized SKOV-3 cells and β -glucan-primed neutrophils or monocytes and wells containing iC3b-

opsonized SKOV-3 cells and non- β -glucan-primed neutrophils or monocytes. For some experiments, Syk kinase inhibitor piceatannol (Sigma-Aldrich) or PI3K inhibitor LY294002 (Calbiochem) was added to the cytotoxicity assay. Human monocytes were isolated using Monocyte Isolation Kit II (Miltenyi Biotec). The CD14⁺ monocytes were >95% as assessed by flow cytometry.

Dual ligation of CR3 and measurement of Syk phosphorylation and PI3K activity

Human neutrophils were stimulated with anti-M1/70 mAb (1 μ g/ml), followed with goat anti-rat Ig (5 μ g/ml) in the presence or absence of the 25-kDa active moiety of β -glucan (10 μ g/ml) at 37°C. To detect Syk phosphorylation, cells were stimulated for 30 min and lysed. The supernatants were incubated with a mixture of anti-CR3 mAbs (OKM-1, MN-41, MO-1, LM-2) and 40 μ l of protein A-agarose for 2 h at 4°C. The immunoprecipitates were analyzed on SDS-PAGE gel. Proteins were transferred onto nitrocellulose membranes and blotted with anti-phospho-Syk Ab or anti-Syk Ab (Santa Cruz Biotechnology). The bound Ab was detected using ECL (Cell Signaling Technology). For measurement of PI3K activity, cells were stimulated for 1 h, and aliquots of cell lysates were incubated for 2 h at 4°C with anti-PI3K p85 Ab. Following this, immune complexes were adsorbed onto protein A-agarose for 3 h. PI3K activity was assayed with PI3K ELISA kit (Echelon Biosciences), according to manufacturer's instructions (20).

Statistical analysis

Data were entered into Prism 4.0 (GraphPad) to generate graphs of percentage of fluorescent-positive cells or tumor regression, and Student's *t* test was used to determine the significance of differences between two data sets. Survival curves were created using the Kaplan-Meier method, and statistical analyses of survival curves used a log-rank test.

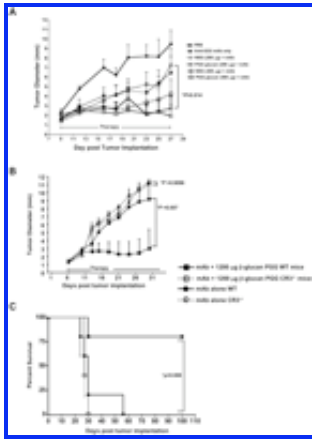
► **Results**

Intravenous administration of β -glucan in combination with anti-tumor mAb enhances antitumor efficacy and improves survival

Our previous studies have demonstrated that daily oral administration of WGP showed antitumor efficacy in a variety of syngeneic tumor models in combination with anti-tumor mAbs (12). In addition, combined therapy with daily i.v.-administered small molecular mass NSG significantly enhanced tumor regression elicited with anti-tumor mAbs (11). In the present experiments, we used β -glucan PGG, a soluble yeast β -glucan with a molecular mass of 150 kDa comprised of a β -D-(1, 2, 3)-linked glucopyranosyl backbone with β -D-(1, 2, 3, 4, 5, 6)-linked β (1, 2, 3) side chains (7). To determine whether β -glucan PGG has a similar therapeutic efficacy, tumor-bearing mice were treated with β -glucan PGG or NSG β -glucan in combination with mAb. Notably, the coadministration of 200 μ g of β -glucan PGG with mAb resulted in significantly more tumor regression than was observed with mAb therapy alone or in combination with 200 μ g of NSG (*p*

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= 0.014; Fig. 1A). The significant tumor regression was achieved using 400 μg of NSG β -glucan daily, as previously demonstrated (11). Therapy with mAb alone showed significant tumor regression, probably due to the high GD2 Ag density of RMA-S cells. However, β -glucan treatment alone did not reach significant tumor regression (data not shown). These results demonstrate that β -glucan PGG has a similar biological activity for tumor therapy. In addition, the lower dose of β -glucan PGG exhibits better tumor regression as compared with small molecular mass NSG β -glucan.



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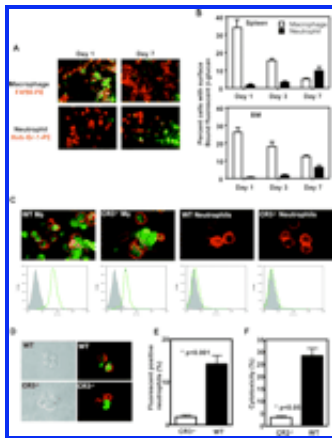
FIGURE 1. The tumoricidal activity of immunotherapy with β -glucan PGG in combination with anti-tumor mAbs. **A**, C57BL/6 mice ($n = 5$) were implanted s.c. with RMA-S cells, and tumors were allowed to form over 9 days before therapy. Mice received PBS, or 14 G2a anti-GD2 ganglioside mAb (100 μg every third day) with or without NSG β -glucan (200 or 400 μg daily) or β -glucan PGG (200 or 400 μg daily) for 3 wk. Tumor measurements were made at the indicated times. **B** and **C**, C57BL/6 mice or CR3-deficient mice ($n = 10$) were implanted s.c. with RMA-S-MUC1 cells, and tumors were allowed to form over 7 days before initiating immunotherapy. Mice were treated with BCP-8 anti-MUC1 mAb (200 μg twice per week) and/or β -glucan PGG (1200 μg twice per week) for a total of 3 wk. Tumor growth (**B**) and survival (**C**) were monitored. Mice were sacrificed when tumors reached 12 mm in diameter. In the current protocol, all mice died through euthanasia due to predefined size limit. Mean values \pm SEM are shown.

Next, we determined whether the efficacy of combined β -glucan PGG with mAb therapy was CR3 dependent, as had been previously shown for other β -glucan preparations. i.v. administration of this polysaccharide twice per week to tumor-bearing mice, in combination with an anti-tumor mAb, almost completely suppressed tumor growth and greatly increased survival, whereas administration of mAb alone had no effect (Fig. 1, **B** and **C**). In contrast, tumor-bearing mice treated with PBS, β -glucan alone (data not shown), or mAb alone (Fig. 1, **B** and **C**) had similar unrestrained tumor growth. This tumor-suppressive effect of β -glucan PGG + mAb was not seen in CR3-deficient (CR3^{-/-}) mice, suggesting that soluble β -glucan PGG-mediated tumor therapy is CR3 dependent. Thus, weekly i.v. β -glucan PGG increases the antitumor efficacy of mAb and improves survival.

CR3-dependent and -independent responses in M ϕ uptake and neutrophil priming with β -glucan

We have shown previously that CR3 has a β -glucan-binding lectin site (14, 21). Soluble β -glucan binds to the lectin site of CR3 and primes circulating phagocytes, permitting cytotoxic degranulation in response to iC3b-opsionized tumor cells that otherwise escape from this mechanism of cell-mediated cytotoxicity. To investigate in vivo CR3 priming by soluble β -glucan, β -glucan PGG was labeled with fluorescein DTAF and

given to mice by i.v. injection in a manner similar to the prior tumor therapy protocol. The in vivo CR3 priming by β -glucan appears to occur through an indirect mechanism. One day following administration of fluorescein-labeled β -glucan to mice, the polysaccharide appeared in splenic M ϕ , but not neutrophils (Fig. 2A). However, 7 days following injection, β -glucan-positive M ϕ virtually disappeared from the spleen, while ~10% of neutrophils, in both the spleen and bone marrow, were now found to contain β -glucan (Fig. 2, A and B).



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FIGURE 2. M ϕ accumulate soluble yeast β -glucan and process it to prime neutrophil CR3. *A* and *B*, Mice given fluorescein DTAF-labeled β -glucan PGG (green) i.v. were sacrificed on days 1, 3, and 7. The spleens were frozen sectioned, and slides were stained with F4/80 PE or anti-Gr-1 PE (red). Original magnification, x60. Cells from the spleen or bone marrow (BM) were stained with F4/80 and anti-Gr-1 mAbs and analyzed by flow cytometry. The percentage of cells with surface-bound fluorescent β -glucan was gated on F4/80⁺ or Gr-1⁺ cells ($n = 5$). *C*, Thioglycolate-elicited M ϕ or neutrophils from WT or CR3-deficient (CR3^{-/-}) mice were incubated with DTAF-labeled β -glucan PGG (green). Cells were observed under confocal microscopy and also analyzed by flow cytometry, respectively. Original magnification, x100 (*D*). Peritoneal neutrophils from WT and CR3^{-/-} mice receiving DTAF-labeled β -glucan PGG were elicited by thioglycolate injection at day 7 and stained with anti-Gr-1 PE (red). Original magnification, x100. *E*, Neutrophils described in *D* were stained with anti-Gr-1 mAb and analyzed by flow cytometry. The percentage of cells with surface-bound fluorescent β -glucan was gated on Gr-1⁺ cells ($n = 5$). *F*, Neutrophils from *D* were assayed for cytotoxicity using iC3b-opsonized RMA-S-MUC1 tumor cells as targets, as described in *Materials and Methods*.

These data suggested that in vivo assortment of β -glucan involves initial M ϕ uptake, followed by the release of β -glucan to neutrophils. To determine the possible importance of CR3 in these events, thioglycolate-elicited peritoneal M ϕ and neutrophils from WT and CR3^{-/-} mice were harvested and incubated with fluorescein-labeled β -glucan. As shown in Fig. 2C, M ϕ from WT and CR3^{-/-} mice had comparable uptake of β -glucan, as assessed by both fluorescence microscopy and FACS analysis, suggesting that the uptake of intact β -glucan by M ϕ is CR3 independent. The uptake of labeled β -glucan was blocked by a 10-fold excess of unlabeled β -glucan (data not shown). Interestingly, the labeled β -glucan did not bind to the neutrophils in these preparations, suggesting that the β -glucan that bound to neutrophils in vivo 7 days following administration (Fig. 2A) might be a modified form of the parent β -glucan, perhaps arising from processing of the original material by M ϕ and subsequent release of the β -glucan fragments to neutrophils. To further explore the importance of CR3 in the priming of neutrophils per se, we examined peritoneal neutrophils,

elicited by thioglycolate, from WT and CR3^{-/-} mice 7 days after administration of labeled β-glucan. Neutrophils from WT, but not CR3^{-/-}, mice exhibited β-glucan binding by both fluorescence microscopy (Fig. 2D) and FACS analysis (Fig. 2E). Furthermore, these WT neutrophils with surface-bound β-glucan were capable of killing iC3b-opsonized RMA-S-MUC1 tumor cells. In contrast, granulocytes from WT mice that had not been given β-glucan killed <5% of iC3b-coated tumor cells (data not shown). The requirement for CR3 was confirmed by absence of tumor killing by neutrophils from CR3^{-/-} mice (Fig. 2F). Therefore, uptake of parent β-glucan by Mφ does not require CR3. However, these results suggested that degradation fragments of the 150-kDa β-glucan might be released from Mφ and bound by WT, but not CR3^{-/-} neutrophils, with the former then being enabled to kill iC3b-opsonized tumor cells.

The 25-kDa β-glucan moiety, but not the parent compound, mediates its biological functions

Our data suggest that i.v.-administrated β-glucan PGG is processed by Mφ to release an active fragment that primes neutrophils for antitumor cytotoxicity in a CR3-dependent manner. In an attempt to identify the nature of this putative active fragment, we used an in vitro Mφ culture system in which resident peritoneal Mφ were exposed to fluorescein-labeled intact β-glucan. Long-term incubation resulted in the appearance of a β-glucan fragment with an approximate molecular size of 25 kDa by high resolution HPLC. Further composition and linkage analysis demonstrated that the 25-kDa active moiety of β-glucan is predominately a linear polymer with β-1,3 backbone and minor branching (9.1%). This fragment, but not the parent β-glucan, bound directly to mouse and human neutrophils or CR3-transfected Chinese hamster ovary cells (Fig. 3, A, B, and D). The binding of this 25-kDa β-glucan fragment by mouse neutrophils was CR3 dependent (Fig. 3C). Interestingly, this 25-kDa β-glucan fragment had a higher binding affinity to human neutrophils as compared with mouse neutrophils, and this binding was saturable (Fig. 3D). Thus, it appears that the 25-kDa β-glucan fragment, but not the 150-kDa parent molecule, binds to CR3 on neutrophils and mediates antitumor cytotoxic effects.

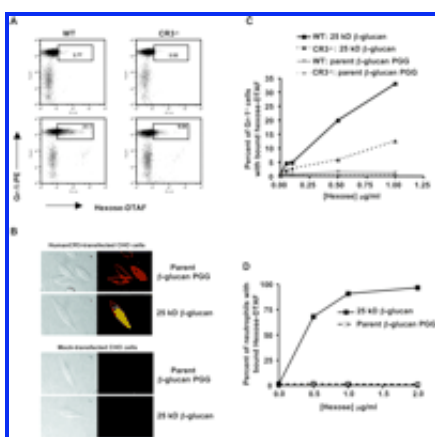


FIGURE 3. An excreted 25-kDa β-glucan fragment binds to neutrophil CR3 (A). Peritoneal neutrophils from WT and CR3^{-/-} mice were stained with DTAF-labeled β-glucan PGG (*upper panel*) or with DTAF-labeled 25-kDa active moiety released from Mφ culture (*lower panel*). B, Human CR3-transfected or mock-transfected Chinese hamster ovary cells (provided by Dr. T. A. Springer, Harvard Medical School, Boston, MA) were cultured in glass plates and stained with anti-CR3 PE (red) and DTAF-labeled parent β-glucan PGG or 25-kDa β-glucan (green). Slides were observed under a Nikon fluorescent microscope. Original magnification, x20. C, Peritoneal neutrophils from WT and CR3^{-/-} mice were stained with various amounts of DTAF-labeled parent β-glucan PGG or 25-kDa β-glucan. The percentage of cells with surface-bound fluorescent β-glucan was gated on Gr-1⁺

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cells. *D*, Purified human peripheral blood neutrophils were stained with various amounts of DTAF-labeled parent β -glucan PGG or 25-kDa β -glucan.

To further characterize the bioactivity of the 25-kDa β -glucan, we exposed human neutrophils to this fragment and assessed the appearance of the activated epitope of CD11b/CD18 (CR3), detected with the mAb CBRM 1/5. The CBRM 1/5 neoepitope is located within the N terminus of CD11b near the I domain, is not exposed on resting neutrophils (22), but appears following exposure of neutrophils to LPS or other proinflammatory agonists (23, 24). As shown in Fig. 4A, neutrophils stimulated by the 25-kDa β -glucan fragment, but not parent β -glucan, induced CBRM 1/5 expression in a dose-dependent manner. In these experiments, exogenous LPS contamination was controlled by maintaining all buffers with 10 μ g/ml polymyxin B. These data indicated that the 25-kDa β -glucan fragment could affect the CR3-dependent priming of neutrophils and might promote neutrophil killing of iC3b-opsonized targets. Indeed, the 25-kDa β -glucan, but not parent β -glucan, greatly amplified the cytotoxicity of neutrophils against iC3b-opsonized human ovarian carcinoma cells in a dose-dependent fashion (Fig. 4B). Although in murine tumor model neutrophils are the predominate effector cells for β -glucan-mediated tumor regression (13), CR3⁺ monocytes/M ϕ could contribute to tumor regression in human tumors. Human monocytes express similar levels of CR3 on the surface as compared with neutrophils (data not shown). As indicated in Fig. 4C, the 25-kDa β -glucan, but not parent β -glucan, significantly elicited the cytotoxicity of monocytes (Fig. 4C). These results suggest that the 25-kDa β -glucan moiety may be responsible for mediating the observed in vivo bioactivity of intact β -glucan PGG.

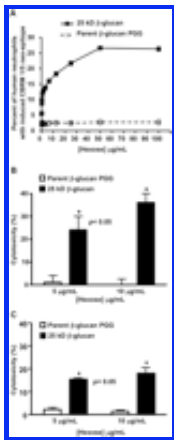


FIGURE 4. Active moiety of 25-kDa β -glucan primes neutrophil CR3 CBRM 1/5 neoepitope induction and permits cytotoxicity. *A*, Human peripheral blood neutrophils were incubated with varying concentrations of parent β -glucan PGG or 25-kDa β -glucan and stained with anti-CBRM 1/5 mAb. *B*, Human neutrophils were cocultured with iC3b-opsonized SKOV-3 tumor cells in the presence of varying concentrations of parent β -glucan PGG or 25-kDa β -glucan for cytotoxicity assay, as described in *Materials and Methods*. The E:T ratio was 20:1. *C*, Human monocytes were cocultured with iC3b-opsonized SKOV-3 tumor cells in the presence of varying concentrations of parent β -glucan PGG or 25-kDa β -glucan for cytotoxicity assay. The E:T ratio was 20:1.

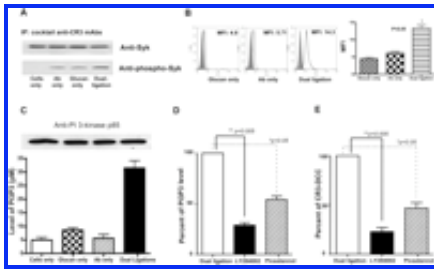
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CR3 dual ligation leads to Syk- and PI3K-dependent cytotoxicity

Previous studies, and our present results, indicate that CR3-dependent cytotoxicity against iC3b-opsonized yeast or tumor cells requires simultaneous ligation of two distinct binding sites in CR3: one for iC3b and the second for β -glucan (25, 26). However, it remains unknown how this dual ligation acts to prompt neutrophil cytotoxicity. Prior investigations of integrin signaling in phagocytes demonstrated a hierarchical activation of the Src family kinase and Syk (27). Syk activation in particular may be crucially important because immobilized anti-CD11b induces a respiratory burst in WT, but not Syk-deficient neutrophils (28). This led us to examine whether CR3 dual ligation might cause Syk activation (i.e., phosphorylation). Human peripheral blood neutrophils were stimulated with an anti-CR3 I domain mAb (M1/70), followed by a secondary Ab to mimic CR3 I domain receptor-ligand binding in the presence or absence of the 25-kDa β -glucan. After stimulation, neutrophils were lysed and the lysates were immunoprecipitated with a mixture of anti-CR3 mAbs. Indeed, we found that Syk phosphorylation was enhanced by dual ligation of CR3 and was coprecipitated with CR3. This was confirmed by Western blot (Fig. 5A) and intracellular anti-phospho-Syk Ab staining assessed by flow cytometry (Fig. 5B).



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FIGURE 5. CR3 dual ligation leads to enhanced Syk phosphorylation, augmented PI3K activity, and cytotoxicity. *A*, Human peripheral blood neutrophils were stimulated with rat anti-human/mouse CR3 I domain mAb M1/70, followed with goat anti-rat secondary Ab with or without 25-kDa β -glucan or 25-kDa β -glucan alone for 30 min. Cell lysates were immunoprecipitated with a mixture of anti-CR3 mAbs, and the immunoprecipitates were analyzed on SDS-PAGE gel. Proteins were transferred onto nitrocellulose membranes and blotted with anti-Syk Ab or anti-phospho-Syk Ab, respectively. *B*, Human peripheral blood neutrophils were stimulated with 25-kDa β -glucan, M1/70 mAb, following with secondary Ab, or both for 30 min. Cells were fixed, permeabilized, and stained with anti-phospho-Syk mAb or isotype control Ab. Cells were assessed by flow cytometry. Mean fluorescence intensity was compared in each stimulation condition. Three independent experiments were performed to acquire the mean \pm SD. *C*, Human peripheral blood neutrophils were stimulated with M1/70 mAb, followed with secondary Ab with or without 25-kDa β -glucan or 25-kDa β -glucan alone for 1 h. Cell lysates were immunoprecipitated with anti-PI3K p85 mAb. The immunoprecipitates were analyzed on SDS-PAGE gel and blotted with anti-PI3K p85 mAb. The immunoprecipitates were also measured for PI3K activity by ELISA. The PI3K activity was represented as the level of phosphatidylinositol(3,4,5)P3 (PI3P3). *D*, Human peripheral blood neutrophils were stimulated with M1/70 mAb, followed with secondary Ab with 25-kDa β -glucan

in the presence or absence of PI3K inhibitor LY294002 (50 μM) and/or Syk kinase inhibitor piceatannol (25 μM) for 1 h. Cells were immunoprecipitated with anti-PI3K p85 mAb, and PI3K activity was measured by ELISA. The PI3K activity was arbitrarily set as 100% for neutrophils stimulated with dual ligation (31.45 \pm 3.8 pM). Percentage of PI3K activity was generated by PI3K activity from inhibitor-treated cells divided by that from dual ligation-stimulated cells (9.05 \pm 1.14 pM for LY294002 and 17.1 \pm 1.5 pM for piceatannol, respectively). Three independent experiments were performed to obtain the absolute levels (mean \pm SD). *E*, Human neutrophils were cocultured with iC3b-opsonized SKOV-3 tumor cells and 25-kDa β -glucan in the presence or absence of PI3K inhibitor LY294002 (50 μM) and/or Syk kinase inhibitor piceatannol (25 μM). The E:T ratio was 20:1. The cytotoxicity was arbitrarily set up as 100% for neutrophils stimulated with 25-kDa β -glucan (35.7 \pm 3.61%). Percentage of CR3-dependent cellular cytotoxicity was generated by cytotoxicity from inhibitor-treated group divided by that from nontreated group (7.88 \pm 1.39% for LY294002 vs 16.15 \pm 2.68% for piceatannol, respectively). Three independent experiments were performed to obtain the absolute levels (mean \pm SD).

PI3K is a family of enzymes that generate specific phosphoinositides that serve as mediators of intracellular signaling (29, 30) and, perhaps, triggering of the respiratory burst in human neutrophils (31). Recent studies indicate that phosphorylated Syk may bind the p85 regulatory subunit of PI3K, perhaps promoting the activity of the latter (32). This suggested that Syk phosphorylation mediated by CR3 dual ligation might lead to enhanced PI3K activity. Indeed, following CR3 dual ligation, we observed increased PI3K activity compared with Ab or glucan stimulation only (Fig. 5C). In further support of the activation of PI3K by phospho-Syk, we observed that the Syk kinase inhibitor, piceatannol (25 μM), significantly blocked this increase in PI3K activity (Fig. 5D). This particular mechanism of signaling appears to be crucial because both the PI3K inhibitor, LY294002 (50 μM), and the Syk kinase inhibitor, piceatannol (25 μM), potentially blocked dual ligation-mediated cytotoxicity (Fig. 5E). Abrogation of cytotoxicity was proportional to the inhibition of PI3K activity mediated by either inhibitor (Fig. 5, D vs E) and a higher dose of the PI3K inhibitor, LY294002 (100 μM), completely abrogated β -glucan-mediated cytotoxicity (data not shown). Therefore, we conclude that the dual ligation of CR3 leads to Syk phosphorylation and downstream PI3K activation and subsequently CR3-dependent cytotoxicity.

► Discussion

Previously published in vitro experiments suggested that small molecular mass

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soluble β -glucans mediated neutrophil, $M\phi$, and NK cell CR3-dependent cellular cytotoxicity of iC3b-opsonized tumors (25, 33, 34). Many of these small molecular mass soluble β -glucans demonstrated in vivo efficacy, but were observed to have very brief in vivo $t_{1/2}$, thus requiring daily i.v. dosing (10, 15). Particulate yeast WGP β -glucan (12) and large molecular mass soluble β -glucan PGG have since been observed to have in vivo antitumor efficacy, but not the capacity to mediate in vitro cytotoxicity. To that end, it was hypothesized that these larger β -glucan compounds were processed in vivo to release a bioactive moiety capable of priming leukocyte CR3 for cytotoxicity of iC3b-opsonized tumor cells. The work presented in this study demonstrates that β -glucan PGG is processed by $M\phi$ in a manner akin to the processing of WGP (12). Furthermore, we show that this activity is ascribed to a 25-kDa β -glucan fragment processed and released by $M\phi$. This active moiety, along with CR3 I domain ligand, activates CR3-Syk-PI3K signaling pathway, leading to CR3-dependent cellular cytotoxicity.

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i.v.-administered β -glucan was found to be taken up predominately by $M\phi$, and this process was CR3 independent. It is likely that $M\phi$ may use other receptors to capture soluble β -glucan PGG. For example, dectin-1 has been described as zymosan glucan receptor (35). However, neutrophils express dectin-1 (36), but fail to bind to β -glucan (Fig. 2C), suggesting that $M\phi$ may use other receptors for β -glucan uptake (37). Nevertheless, this observation recalls the function of $M\phi$ in host defense and the capture and presentation of Ag, and may account for the indicated efficiency of β -glucan uptake. Indeed, the digestion of β -glucan PGG in vivo was shown to be very rapid, as demonstrated by the inability to detect splenic $M\phi$ containing β -glucan 7 days following i.v. administration. The inability to detect splenic β -glucan-binding $M\phi$ may be due to the significant down-regulation of CR3 on $M\phi$ that uptake β -glucan PGG (our unpublished observation). It appears that the in vivo priming of neutrophil CR3 is relatively inefficient in that only ~10% of the margined pool of neutrophils is primed for CR3-dependent cytotoxicity. However, this seemingly inefficient priming results in a robust effector population capable of mediating in vivo tumor regression and survival. In addition, 10% may represent an internal threshold to limit the unchecked activation of neutrophils that may lead to autoimmunity or unnecessary inflammation.

Ex vivo and in vitro processing of β -glucan PGG by resident peritoneal $M\phi$ yielded soluble moieties of β -glucan of ~25 kDa. These moieties demonstrated comparable bioactivity as measured by the ability to bind to murine neutrophils in a CR3-dependent manner and the ability to prime human neutrophil CR3 as detected by an activation neoepitope of human CR3 (CD11b) and elicit CR3-dependent cellular cytotoxicity. It is worth noting that although the 25-kDa β -glucan has a high binding affinity to human neutrophils, only 25% of neutrophils are detected for CBRM 1/5 neoepitope expression. In addition, the concentration of the 25-kDa β -glucan for the requirement of CBRM 1/5 CR3 neoepitope induction is significantly higher than that for neutrophil binding (Figs. 3D and 4A). It has been reported that neoepitope expression of CBRM 1/5 can be induced by chemoattractants or phorbol ester (PMA) and requires the conformational change of CR3 (22, 38). PMA induces CBRM neoepitope on 30% of CR3 molecules ($57,200 \pm 1,300$ sites/cell), whereas fMLP induces CBRM 1/5 on 10% of CR3 molecules ($13,200 \pm 1,400$ sites/cell). The expression levels of

CBRM 1/5 neopeptide (sites/cell) vary depending on the different stimuli. Although 100% neutrophils bind β -glucan via lectin-like domain of CR3 (39), the expression levels of CBRM 1/5 (sites/cell) are lower as compared with PMA stimulation. Therefore, the intensity of CBRM 1/5 expression as detected by FITC-labeled Ab is relatively low. In addition, the conformational change of CR3 to exposure neopeptide CBRM 1/5 may require cross-linking of lectin-like domain, thus requiring higher concentration of hexose. Nevertheless, our data suggest that the 25-kDa β -glucan, not the parent β -glucan, is necessary and sufficient for CR3-dependent, neutrophil- or monocyte-mediated cytotoxicity against iC3b-opsonized tumor cells. These results demonstrate for the first time the bioactive moiety of yeast β -glucan that may be responsible for mediating the observed *in vivo* effects of β -glucan for tumor therapy in combination with anti-tumor mAb. Furthermore, the demonstrated *in vivo* efficacy of weekly dosing of β -glucan suggests that β -glucan PGG is indeed a reservoir of bioactive material and that M ϕ may slowly release the bioactive moiety to prime neutrophils. A lower dose of β -glucan PGG shows better tumor regression than daily dosing of small molecular mass NSG β -glucan, further suggesting the benefits to administration of β -glucan PGG for enhanced anti-tumor mAb tumor therapy. This phenomenon may be important because neutrophils have short circulating $t_{1/2}$. Therefore, slow release of bioactive β -glucan would be expected to maintain a constant supply of primed neutrophils despite their high turnover rate. In addition, the activation of neutrophils via cytokines, including GM-CSF, has been observed to increase the $t_{1/2}$ of neutrophils by delaying their self-initiated apoptosis through increasing the activity of the PI3K and ERK pathways (40). The bioactive moiety of β -glucan may activate neutrophils to similarly experience a longer *in vivo* $t_{1/2}$, thus necessitating the need to better characterize the signaling pathways activated in neutrophils by this bioactive moiety. This proposed function would be expected to assist in host defense. As M ϕ encounter and phagocytose opsonized or nonopsonized fungal pathogens, the digestion of these fungi results in the systemic release of β -glucans that are detectable in the serum of patients with systemic mycoses. These β -glucans may indeed prime a small pool of neutrophils to engage other iC3b-opsonized fungi for cytotoxicity. In addition, these systemic β -glucans may increase the useful $t_{1/2}$ of neutrophils, thereby maintaining a capable effector population to clear a systemic infection for a longer period of time.

The dual ligation of CR3 triggers enhanced Syk kinase activation and a subsequent PI3K pathway necessary for cytotoxicity. CR3 is one of the most important phagocyte receptors for recognition of microbial pathogens and is responsible for mediating phagocytosis, degranulation, and respiratory bursts in response to iC3b-opsonized bacterial and yeast (41). Polysaccharide priming of CR3 involves a magnesium- and protein tyrosine kinase-dependent conformation change in CD11b that exposes the activation epitope (25). Critical to these functions is a lectin site in CR3 CD11b subunit (α_M) that binds to a polysaccharide for stimulation of cytotoxic reaction. Coprecipitation of phosphorylated Syk with CR3 implies that activation of CD11b may directly recruit Syk kinase in neutrophils. In addition, we presented evidence suggestive of an interaction between the phosphorylated Syk and PI3K pathway as inhibition of Syk kinase abrogated PI3K activity (Fig. 5D). Most importantly, inhibition of the PI3K pathway almost completely ameliorated any CR3 dual ligation-mediated cellular cytotoxicity (Fig. 5E). Previous study also demonstrates the importance

of the PI3K pathway in β -glucan-mediated protective effect in sepsis as inhibition of PI3K eliminates such an effect (42). We believe that dual ligation of the CD11b I domain and the lectin-like domain leads to recruitment of Syk and subsequently initiates the PI3K signaling cascade and permits neutrophil cytotoxicity.

The work presented in this study provides a more complete picture of the mechanisms involved in the antitumor effects of β -glucan in combination with anti-tumor mAbs. Intact β -glucan is first taken up by $M\phi$ and cleaved into a 25-kDa active fragment. This active fragment is evidently released from the $M\phi$, binds to neutrophil CR3, and primes these cells for target killing through signaling events involving both Syk and PI3K. The importance of these observations is that β -glucan is without evident toxicity, and can be orally administered and used in conjunction with existing anti-tumor mAbs to greatly amplify tumor cell killing. We believe this may open new opportunities in the immunotherapy of cancer.

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► Disclosures

Dr. Jun Yan has declared a financial interest in Biothera, whose product was studied in the present work.

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² B.L. and D.J.A. contributed equally to this work. [↑](#)

³ Address correspondence and reprint requests to Dr. Jun Yan, Tumor Immunobiology Program, James

Graham Brown Cancer Center, Delia D. Baxter Research Building, Room 119A, University of Louisville, 580 South Preston Street, Louisville, KY 40202. E-mail address: jun.yan@louisville.edu 

⁴ Abbreviations used in this paper: WGP, whole glucan particle; CR3, C receptor 3; DTAF, dichlorotriazine; I domain, inserted domain; M ϕ , macrophage; NSG, neutral soluble glucan; WT, wild type. 

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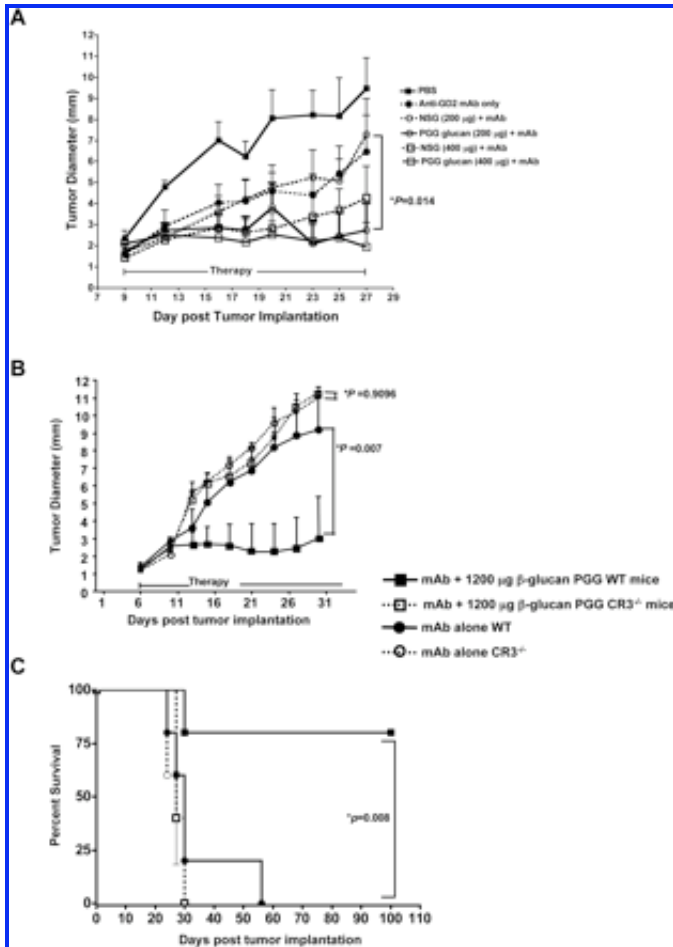


FIGURE 1. The tumoricidal activity of immunotherapy with β -glucan PGG in combination with anti-tumor mAbs. *A*, C57BL/6 mice ($n = 5$) were implanted s.c. with RMA-S cells, and tumors were allowed to form over 9 days before therapy. Mice received PBS, or 14 G2a anti-GD2 ganglioside mAb (100 μ g every third day) with or without NSG β -glucan (200 or 400 μ g daily) or β -glucan PGG (200 or 400 μ g daily) for 3 wk. Tumor measurements were made at the indicated times. *B* and *C*, C57BL/6 mice or CR3-deficient mice ($n = 10$) were implanted s.c. with RMA-S-MUC1 cells, and tumors were allowed to form over 7 days before initiating immunotherapy. Mice were treated with BCP-8 anti-MUC1 mAb (200 μ g twice per week) and/or β -glucan PGG (1200 μ g twice per week) for a total of 3 wk. Tumor growth (*B*) and survival (*C*) were monitored. Mice were sacrificed when tumors reached 12 mm in diameter. In the current protocol, all mice died through euthanasia due to predefined size limit. Mean values \pm SEM are shown.

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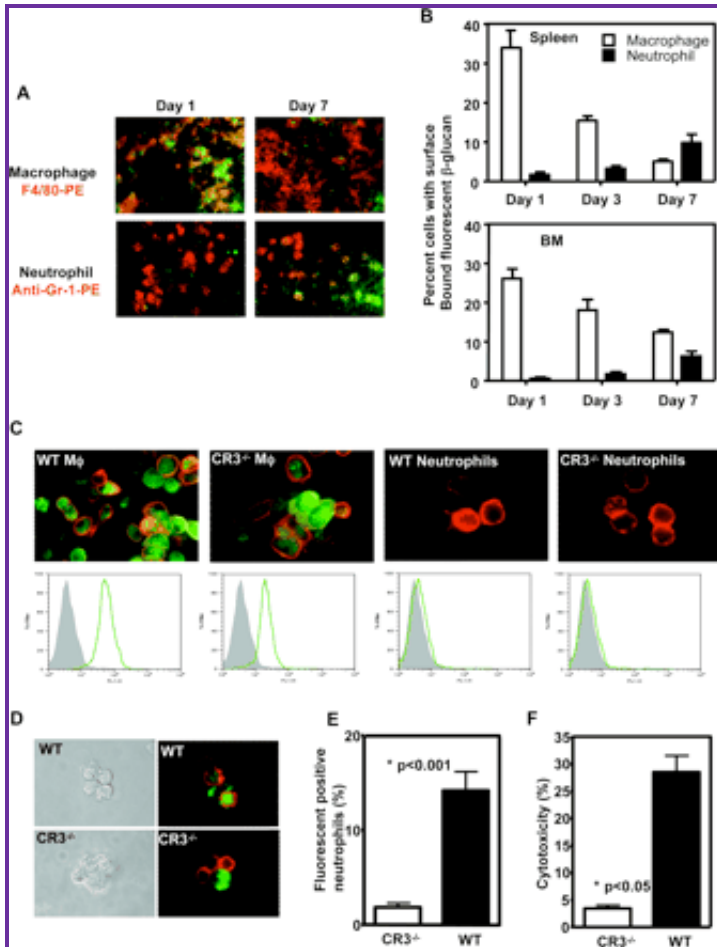


FIGURE 2. M ϕ accumulate soluble yeast β -glucan and process it to prime neutrophil CR3. *A* and *B*, Mice given fluorescein DTAF-labeled β -glucan PGG (green) i.v. were sacrificed on days 1, 3, and 7. The spleens were frozen sectioned, and slides were stained with F4/80 PE or anti-Gr-1 PE (red). Original magnification, x60. Cells from the spleen or bone marrow (BM) were stained with F4/80 and anti-Gr-1 mAbs and analyzed by flow cytometry. The percentage of cells with surface-bound fluorescent β -glucan was gated on F4/80⁺ or Gr-1⁺ cells ($n = 5$). *C*, Thioglycolate-elicited M ϕ or neutrophils from WT or CR3-deficient (CR3^{-/-}) mice were incubated with DTAF-labeled β -glucan PGG (green). Cells were observed under confocal microscopy and also analyzed by flow cytometry, respectively. Original magnification, x100 (*D*). Peritoneal neutrophils from WT and CR3^{-/-} mice receiving DTAF-labeled β -glucan PGG were elicited by thioglycolate injection at day 7 and stained with anti-Gr-1 PE (red). Original magnification, x100. *E*, Neutrophils described in *D*

were stained with anti-Gr-1 mAb and analyzed by flow cytometry. The percentage of cells with surface-bound fluorescent β -glucan was gated on Gr-1⁺ cells ($n = 5$). *F*, Neutrophils from *D* were assayed for cytotoxicity using iC3b-opsonized RMA-S-MUC1 tumor cells as targets, as described in *Materials and Methods*.

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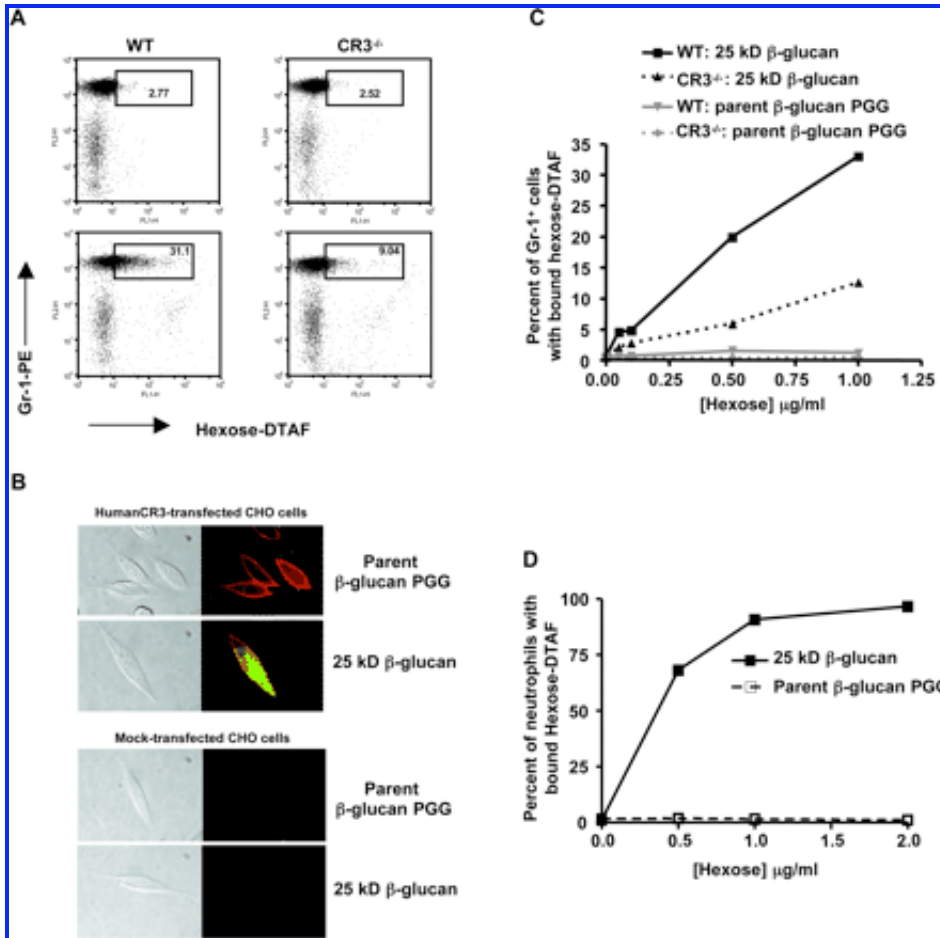


FIGURE 3. An excreted 25-kDa β -glucan fragment binds to neutrophil CR3 (A). Peritoneal neutrophils from WT and CR3^{-/-} mice were stained with DTAF-labeled β -glucan PGG (*upper panel*) or with DTAF-labeled 25-kDa active moiety released from M ϕ culture (*lower panel*). B, Human CR3-transfected or mock-transfected Chinese hamster ovary cells (provided by Dr. T. A. Springer, Harvard Medical School, Boston, MA) were cultured in glass plates and stained with anti-CR3 PE (red) and DTAF-labeled parent β -glucan PGG or 25-kDa β -glucan (green). Slides were observed under a Nikon fluorescent microscope. Original magnification, x20. C, Peritoneal neutrophils from WT and CR3^{-/-} mice were stained with various amounts of DTAF-labeled parent β -glucan PGG or 25-kDa β -glucan. The percentage of cells with surface-bound fluorescent β -glucan was gated on Gr-1⁺ cells. D, Purified human peripheral blood neutrophils were stained with various amounts of DTAF-labeled parent β -glucan PGG or 25-kDa β -glucan.

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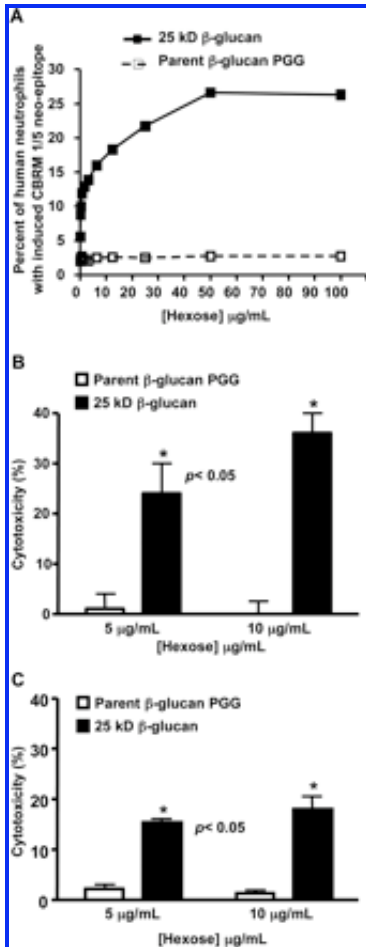


FIGURE 4. Active moiety of 25-kDa β -glucan primes neutrophil CR3 CBRM 1/5 neopeptide induction and permits cytotoxicity. *A*, Human peripheral blood neutrophils were incubated with varying concentrations of parent β -glucan PGG or 25-kDa β -glucan and stained with anti-CBRM 1/5 mAb. *B*, Human neutrophils were cocultured with iC3b-opsonized SKOV-3 tumor cells in the presence of varying concentrations of parent β -glucan PGG or 25-kDa β -glucan for cytotoxicity assay, as described in *Materials and Methods*. The E:T ratio was 20:1. *C*, Human monocytes were cocultured with iC3b-opsonized SKOV-3 tumor cells in the presence of varying concentrations of parent β -glucan PGG or 25-kDa β -glucan for cytotoxicity assay. The E:T ratio was 20:1.

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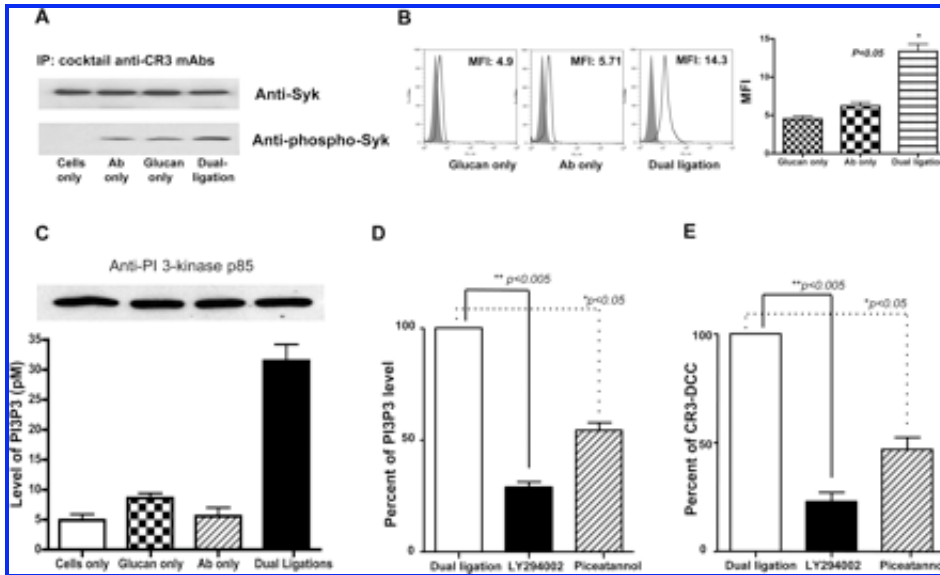


FIGURE 5. CR3 dual ligation leads to enhanced Syk phosphorylation, augmented PI3K activity, and cytotoxicity. *A*, Human peripheral blood neutrophils were stimulated with rat anti-human/mouse CR3 I domain mAb M1/70, followed with goat anti-rat secondary Ab with or without 25-kDa β -glucan or 25-kDa β -glucan alone for 30 min. Cell lysates were immunoprecipitated with a mixture of anti-CR3 mAbs, and the immunoprecipitates were analyzed on SDS-PAGE gel. Proteins were transferred onto nitrocellulose membranes and blotted with anti-Syk Ab or anti-phospho-Syk Ab, respectively. *B*, Human peripheral blood neutrophils were stimulated with 25-kDa β -glucan, M1/70 mAb, following with secondary Ab, or both for 30 min. Cells were fixed, permeabilized, and stained with anti-phospho-Syk mAb or isotype control Ab. Cells were assessed by flow cytometry. Mean fluorescence intensity was compared in each stimulation condition. Three independent experiments were performed to acquire the mean \pm SD. *C*, Human peripheral blood neutrophils were stimulated with M1/70 mAb, followed with secondary Ab with or without 25-kDa β -glucan or 25-kDa β -glucan alone for 1 h. Cell lysates were immunoprecipitated with anti-PI3K p85 mAb. The immunoprecipitates were analyzed on SDS-PAGE gel and blotted with anti-PI3K p85 mAb. The immunoprecipitates were also measured for PI3K activity by ELISA. The PI3K activity was represented as the level of phosphatidylinositol(3,4,5)P3 (PI3P3). *D*, Human peripheral blood neutrophils were stimulated with M1/70 mAb, followed with secondary Ab with 25-kDa β -glucan in the presence or absence of PI3K inhibitor LY294002 (50 μ M) and/or Syk kinase inhibitor piceatannol (25 μ M) for 1 h. Cells were immunoprecipitated with anti-PI3K p85 mAb, and PI3K activity was measured by ELISA. The PI3K activity was arbitrarily set as 100% for neutrophils stimulated with dual ligation (31.45 \pm 3.8 pM). Percentage of PI3K activity was generated by PI3K activity from inhibitor-treated cells divided by that from dual ligation-

stimulated cells (9.05 ± 1.14 pM for LY294002 and 17.1 ± 1.5 pM for piceatannol, respectively). Three independent experiments were performed to obtain the absolute levels (mean \pm SD). *E. Coli* were cocultured with iC3b-opsonized SKOV-3 tumor cells and 25-kDa β -glucan in the presence or absence of PI3K inhibitor LY294002 ($50 \mu\text{M}$) and/or Syk kinase inhibitor piceatannol ($25 \mu\text{M}$). The E:T ratio was 20:1. The cytotoxicity was arbitrarily set up as 100% for neutrophils stimulated with 25-kDa β -glucan ($35.7 \pm 3.61\%$). Percentage of CR3-dependent cellular cytotoxicity was generated by cytotoxicity from inhibitor-treated group divided by that from nontreated group ($7.88 \pm 1.39\%$ for LY294002 vs $16.15 \pm 2.68\%$ for piceatannol, respectively). Three independent experiments were performed to obtain the absolute levels (mean \pm SD).

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