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**Citation:** Bulmer, Mark S et al. "Targeting an antimicrobial effector function in insect immunity as a pest control strategy." Proceedings of the National Academy of Sciences 106.31 (2009): 12652-12657. © 2010 National Academy of Sciences

**As Published:** <http://dx.doi.org/10.1073/pnas.0904063106>

**Publisher:** United States National Academy of Sciences

**Persistent URL:** <http://hdl.handle.net/1721.1/52551>

**Version:** Final published version: final published article, as it appeared in a journal, conference proceedings, or other formally published context

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# Targeting an antimicrobial effector function in insect immunity as a pest control strategy

Mark S. Bulmer<sup>a,1,2,3</sup>, Ido Bachelet<sup>b,1</sup>, Rahul Raman<sup>b</sup>, Rebeca B. Rosengaus<sup>a,4</sup>, and Ram Sasisekharan<sup>b,3,4</sup>

<sup>a</sup>Department of Biology, Northeastern University, Boston, MA 02115; and <sup>b</sup>Harvard–MIT Division of Health Sciences and Technology, Department of Biological Engineering, Koch Institute for Integrative Cancer Research, Massachusetts Institute of Technology, Cambridge, MA 02139

Communicated by Gerald N. Wogan, Massachusetts Institute of Technology, Cambridge, MA, April 13, 2009 (received for review January 27, 2009)

**Insect pests such as termites cause damages to crops and man-made structures estimated at over \$30 billion per year, imposing a global challenge for the human economy. Here, we report a strategy for compromising insect immunity that might lead to the development of nontoxic, sustainable pest control methods. Gram-negative bacteria binding proteins (GNBPs) are critical for sensing pathogenic infection and triggering effector responses. We report that termite GNBPs (tGNBP-2) shows  $\beta(1,3)$ -glucanase activity previously unknown in animal immunity and is a pleiotropic pattern recognition receptor and an antimicrobial effector protein. Termites incorporate this protein into the nest building material, where it functions as a nest-embedded sensor that cleaves and releases pathogenic components, priming termites for improved antimicrobial defense. By means of rational design, we present an inexpensive, nontoxic small molecule glycomimetic that blocks tGNBP-2, thus exposing termites *in vivo* to accelerated infection and death from specific and opportunistic pathogens. Such a molecule, introduced into building materials and agricultural methods, could protect valuable assets from insect pests.**

$\beta(1,3)$ -glucanase | Gram-negative bacteria binding proteins | pattern recognition receptor | termites | social insect immunity

Insect immune systems are simple, efficient, and still enigmatic (1–3). Somatic Ig hypervariability has been observed in *Anopheles* and *Drosophila* (4, 5) and may represent ancestral versions of adaptive immunity, although its evolutionary and functional significance in this context is not clear. Insects employ other, well-characterized mechanisms. Among these are pattern recognition receptors, which recognize molecular determinants unique to different classes of pathogenic microorganisms (1–3).

Gram-negative bacteria binding proteins (GNBPs) are a class of conserved receptors (6, 7) that signal the presence of pathogens once they enter the hemocoel (8). Insect GNBPs contain regions with significant homology to bacterial  $\beta$ -glucanases, especially  $\beta(1,3)$ - and  $\beta(1,3)$ -(1,4)-glucanases (6, 9–11) and likely represent evolutionary descendants of enzymes originally serving homeostatic or digestive functions. Several peptidoglycan recognition proteins, members of a different receptor group in mammals and insects, were shown to be in fact active amidases that either initiate protective signaling cascades or are directly bactericidal (12). GNBPs are believed to have lost enzymatic activity and thus serve only as pattern recognition receptors (9, 10, 13).

Here, we show that a termite GNBPs demonstrates  $\beta(1,3)$ -glucanase activity, serving a critical effector function in antimicrobial defense. By analysis of the structure–function relationships of this protein, we present a small molecule glycomimetic that is capable of blocking it, thus suppressing the insect's immune system and exposing it to attacks from specific and opportunistic pathogens. This molecule represents an inexpensive, nontoxic, and environmentally safe alternative to toxic pesticide chemicals currently in global use.

## Results and Discussion

**Termites Express  $\beta(1,3)$ -Glucanase Activity.** In a previous study (6), we reported that termite GNBPs were positively selected fol-

lowing a single duplication event before the divergence of *Mastotermes*, the most ancient lineage of the Isoptera. Adaptive evolution in termite GNBPs appears to have been driven by a coevolutionary arms race and shifts in habitat that have likely exposed termites to new groups of pathogenic microorganisms (6, 14, 15). However, our sequence analysis of GNBPs from various termite lineages showed that the critical residues involved in the  $\beta(1,3)$ -glucanase activity remained surprisingly intact. Interestingly, the catalytic site appears to have been maintained in the GNBPs of several other insects, and their phylogenetic distribution suggests that GNBPs cluster in discrete groups that have either maintained or lost  $\beta(1,3)$ -glucanase activity (Fig. 1A).

Measurement of  $\beta(1,3)$ -glucanase activity in *Nasutitermes corniger*, performed by electrophoresis on a chromogenic substrate gel, revealed significant activity in body tissues and secretions including salivary glands and cuticular washes (Fig. 1B). Other termite species (*Zootermopsis augusticollis*, *Cryptotermes secundus*, and *Rubricaps flavipes*) also showed robust  $\beta(1,3)$ -glucanase activity. This activity was demonstrated by all castes. Other insects, such as *Galleria mellonella* and *Drosophila melanogaster* (Fig. 1B), had no activity. Still, this activity is not limited to termites as suggested by our sequence analysis and as recently reported for several pest species (16–18).

The fungal entomopathogen *Metarhizium anisopliae* is a natural termite pathogen and is currently being developed for the biological control of termites and other insect pests (19). *M. anisopliae* conidia treated with  $\beta(1,3)$ -glucanases purified from either *Helix pomatia* or *Bacillus subtilis* collapsed due to turgor pressure loss and leakage of intracellular components. Similarly, conidia treated with a termite protein size-exclusion fraction coinciding with peak  $\beta(1,3)$ -glucanase activity likewise collapsed, and their cell volume decreased by 25% (Fig. 1C, cell volume reduction shown in *Inset*).

## Termite GNBPs Is an Active $\beta(1,3)$ -Glucanase Induced by Pathogenic Patterns.

To associate a termite GNBPs with the observed activity, antibodies were raised against termite GNBPs-1 and -2 (tGNBP-1 and tGNBP-2, respectively). Native, tGNBP-2 was successfully isolated and purified by immunoaffinity chromatography from both termite extract and nests built from various materials (Fig. 2A) and exhibited robust  $\beta(1,3)$ -glucanase activity (Fig. 2B). No equivalent proteins were precipitated from soil or wood.

Author contributions: M.S.B., I.B., R.B.R., and R.S. designed research; M.S.B., I.B., and R.R. performed research; R.B.R. and R.S. contributed new reagents/analytic tools; M.S.B., I.B., R.R., R.B.R., and R.S. analyzed data; and M.S.B., I.B., R.B.R., and R.S. wrote the paper.

The authors declare no conflict of interest.

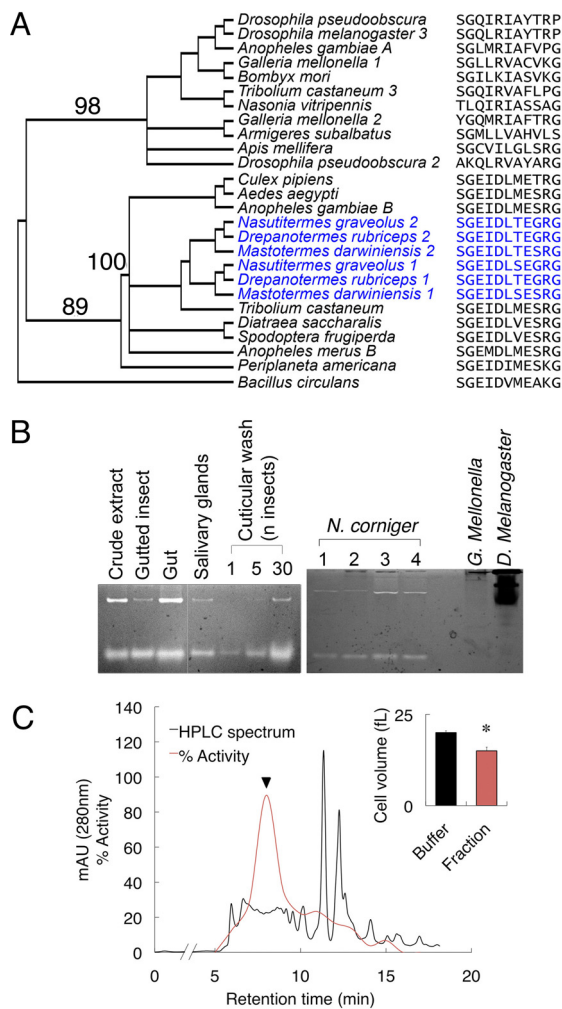
<sup>1</sup>M.S.B. and I.B. contributed equally to this work.

<sup>2</sup>Present address: Department of Biological Sciences, Towson University, Towson, MD 21252.

<sup>3</sup>To whom correspondence may be addressed. E-mail: mbulmer@towson.edu or rams@mit.edu.

<sup>4</sup>R.B.R. and R.S. contributed equally to this work.

This article contains supporting information online at [www.pnas.org/cgi/content/full/0904063106/DCSupplemental](http://www.pnas.org/cgi/content/full/0904063106/DCSupplemental).



**Fig. 1.** Termites exhibit an unusual  $\beta(1,3)$ -glucanase activity. (A) Distance tree of insect GNBP sequences rooted with *B. circulans*  $\beta(1,3)$ -glucanase. Numbers depict bootstrap values for basal lineages. Glucanase motif sequences are adjacent to species name. Termite species appear in blue. Numbers following species name indicate GNBP-1, -2, etc. (B)  $\beta(1,3)$ -Glucanase activity in termite tissues and cuticular washes, assayed on Carboxymethyl Curdlan Remazol Brilliant Blue gels (lanes: 1, soldier; 2, large worker; 3, large worker; 4, 10 workers; 5–7, 1, 5, and 30 large workers, respectively) (Left). Comparison of activity in extracts from termites (lanes: 1, *Nasutitermes corniger* collected from Florida; 2–4, *N. corniger* collected from different colonies in Panama), *Drosophila melanogaster* and *Galleria mellonella* (Right). (C) Activity profile of fractionated termites separated by HPLC. (Inset) Cytotoxic effect of the peak active fraction (marked with an arrowhead) from termites on *Metarhizium anisopliae* conidia measured by flow cytometry as the effect on cell volume in femtoliters (\*,  $P < 0.05$  vs. buffer).

tGNBP-2 was detected in termite HPLC fractions displaying peak  $\beta(1,3)$ -glucanase activity (Fig. S1) and was found to be expressed on the surfaces of two distinct hemocyte populations, granular hemocytes (Electronic Volume<sup>lo</sup>/Side Scatter<sup>hi</sup>) and large, less dense hemocytes (Electronic Volume<sup>hi</sup>/Side Scatter<sup>lo</sup>, presumably plasmatocytes) (Fig. 2C). The hydrophobic tail of tGNBP-2 is highly homologous to the tail of *Drosophila* GNBP (20) and therefore likely serves as a GPI anchor. Existence in both soluble and membrane-associated forms is common among pattern receptors including mammalian CD14 (21) and various insect proteins (22).

The presence of active tGNBP-2 in nest material indicates that termites incorporate but also maintain the levels of this protein in the nest structure. This observation points to a possible

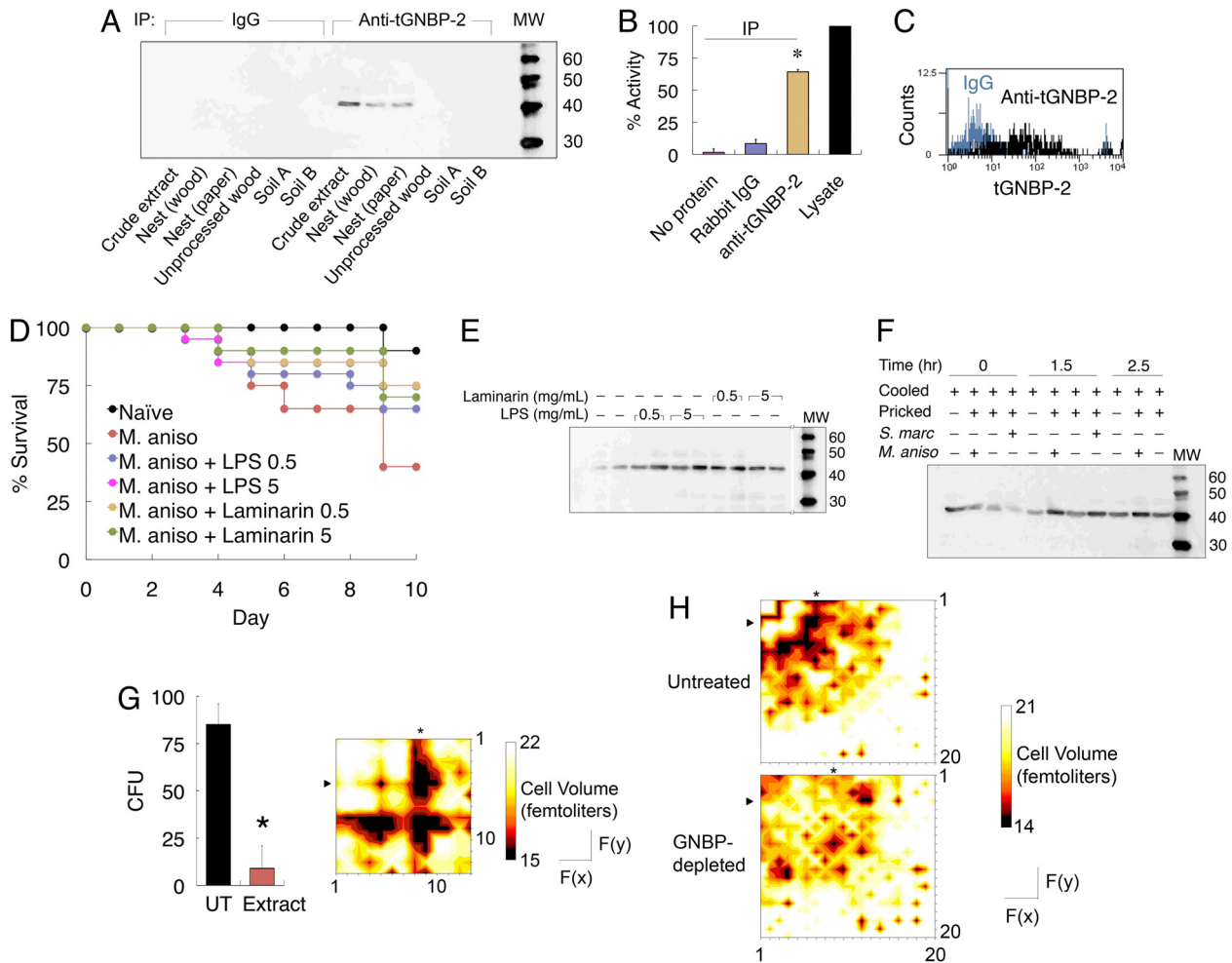
mechanism wherein tGNBP-2 functions as a nest-embedded sensor that cleaves and releases pathogenic components, which then prime termites for improved antimicrobial immunity. Indeed, termites exposed to either Gram-negative bacteria-derived LPS or an algae-derived  $\beta(1,3)$ -D-glucan show improved resistance to *M. anisopliae* infection (Fig. 2D). Analysis of tGNBP-2 expression revealed significant up-regulation following exposure to either molecular component (Fig. 2E). Moreover, infection of termites with either *Serratia marcescens* or *M. anisopliae* also induced the expression of tGNBP-2 as early as 1.5 h postinfection, whereas control groups for stress caused by cooling and pin-pricking remained close to baseline levels (Fig. 2F). This finding is in agreement with a previously reported *Drosophila* GNBP (20) and shows that tGNBP-2 is pattern specific rather than stress induced. The use of tGNBP-2 as an external defense system is likely to have been instrumental in the evolution of complex societies with elaborate nest architectures. Moreover, evolution of social behaviors, such as the increased mutual grooming observed following exposure to fungal pathogens (23), may have been directed by the protective function of salivary tGNBP-2.

**tGNBP-2 Is an Antimicrobial Effector Protein.** Two important questions were: (a) Is the  $\beta(1,3)$ -glucanase activity of tGNBP-2 an effector activity, and (b) does this activity critically contribute to the total effector capacity of the termite immune system, or is it redundant to additional mechanisms? Crude termite extract, representing the total effector capacity, is cytotoxic to *Metarhizium* conidia (Fig. 2G Left). To observe this at a higher resolution, the extract was fractionated into either 13 or 20 size fractions crossed in  $13 \times 13$  or  $20 \times 20$  matrices, respectively, and the cytotoxicity of every fraction combination toward *Metarhizium* was evaluated by flow cytometric analysis of conidia cell volume.

Combining tGNBP-2<sup>+</sup> fractions with fractions corresponding to  $\approx 5,000$  Da molecules (Fig. 2G Right) resulted in significant synergistic cytotoxicity compared with that exerted by tGNBP-2 alone. This synergistic effect was abolished by depletion of tGNBP-2 (Fig. 2H). One possible explanation for this intriguing finding is that tGNBP-2 cooperates with small antimicrobial peptides such as termicin and spinigerin (24–26) by compromising cell wall integrity and enhancing peptide penetration into the cell, a mechanism used by plant  $\beta(1,3)$ -glucanases (27). These antimicrobial peptides are constitutively expressed at high levels in termites, and mass spectrometric analysis suggested the presence of these peptides by size (Fig. S2). Termicins are also secreted by salivary glands (26). This combinatorial mapping demonstrates that tGNBP-2 is a critical component of the termite antimicrobial potential.

**Structure–Function Analysis of the Pattern Recognition and Enzymatic Activity of tGNBP-2.** To examine whether tGNBP-2 represents a functional pattern recognition receptor, we quantified the interactions between this protein and a range of intact termite pathogens. Isolated tGNBP-2 exhibited binding to both Gram-negative bacteria and fungi, with significantly higher affinity to bacteria than to fungi (Fig. 3A).

The structural basis of this dual function was studied using a homology-based model where  $\beta(1,3)$ -D-glucan and eritoran (an LPS analog) are docked into tGNBP-2. This model suggests that  $\beta(1,3)$ -D-glucans are bound and catalyzed at the intact glucanase region, whereas a distinct hydrophobic patch upstream to it binds LPS (Fig. 3B). Homologous patches have been shown to bind LPS in other insects (28). The model shows that the  $\beta(1,3)$ -D-glucan is potentially held in place by 6 residues, 5 of which are chemically similar in termite GNBP-2 proteins and in *Bacillus circulans*  $\beta(1,3)$ -glucanase but vary among other insect GNBP (Table S1), providing a structural insight into the enzymatic



**Fig. 2.** tGNBP-2 is an effector  $\beta(1,3)$ -glucanase induced by pathogenic patterns. (A) Immunoprecipitation of tGNBP-2 from termite extracts, nests, unprocessed wood, and soils A and B sampled from different locations. (B)  $\beta(1,3)$ -Glucanase activity of termite immunoprecipitate isolated by rabbit IgG or anti-tGNBP-2 (\*,  $P < 0.05$  vs. no protein and rabbit IgG). (C) Expression of tGNBP-2 on hemocyte surface, measured by flow cytometry. (D) Survival of termites infected with *Metarhizium anisopliae* (*M. aniso*) following immunization with LPS or laminarin at either 0.5 or 5 mg/mL along a course of 10 days (mean of 2 groups,  $n = 12$ /group; all immunized termite groups were  $P < 0.05$  vs. *M. aniso* only, and  $P > 0.05$  vs. naïve termites with the exception of 0.5 mg/mL LPS). (E) tGNBP-2 expression after exposure of termites to either LPS or laminarin at either 0.5 or 5 mg/mL (left and right lanes in each sample represent two independent repeats). (F) Expression of tGNBP-2 after termite infection with *M. anisopliae* (*M. aniso*) or *Serratia marcescens* (*S. marc*). Cooled only (cooled) or cooled and pricked (pricked) termites are shown as controls. (G) Effect of crude termite extract on conidial growth (in CFUs) on potato-dextrose agar plates (\*,  $P < 0.05$  vs. untreated conidia) (Left). Cytotoxicity map of fractionated termites on *M. anisopliae* conidia measured by cell volume in femtoliters (Right).  $F(x), F(y)$  represent fractions 1–13 on either axis. The tGNBP-2<sup>+</sup> fraction is marked by an arrowhead; asterisks mark putative antimicrobial peptides. (H) Loss of cytotoxicity after depletion of tGNBP-2 by antibody precipitation.  $F(x), F(y)$  represent fractions 1–20 on either axis.

activity of tGNBP-2. Interestingly, we found that binding of LPS and catalysis of  $\beta(1,3)$ -D-glucans do not cross-interfere with each other, showing that they are distinct both structurally and functionally (Fig. S3).

**Rational Design of a Glycomimetic tGNBP-2 Blocker.** These findings implicated the  $\beta(1,3)$ -glucanase activity of tGNBP-2 as a critical component in termite antimicrobial defense. We therefore sought a way to block this protein that will target only its unusual  $\beta(1,3)$ -glucanase activity and leave its other parts intact. This requirement along with our aim of designing a pest control strategy highlighted a small molecule approach. We hypothesized that a glycomimetic derivative of the pathogenic patterns recognized by tGNBP-2 would be both a structurally rational and a synthetically feasible blocker.

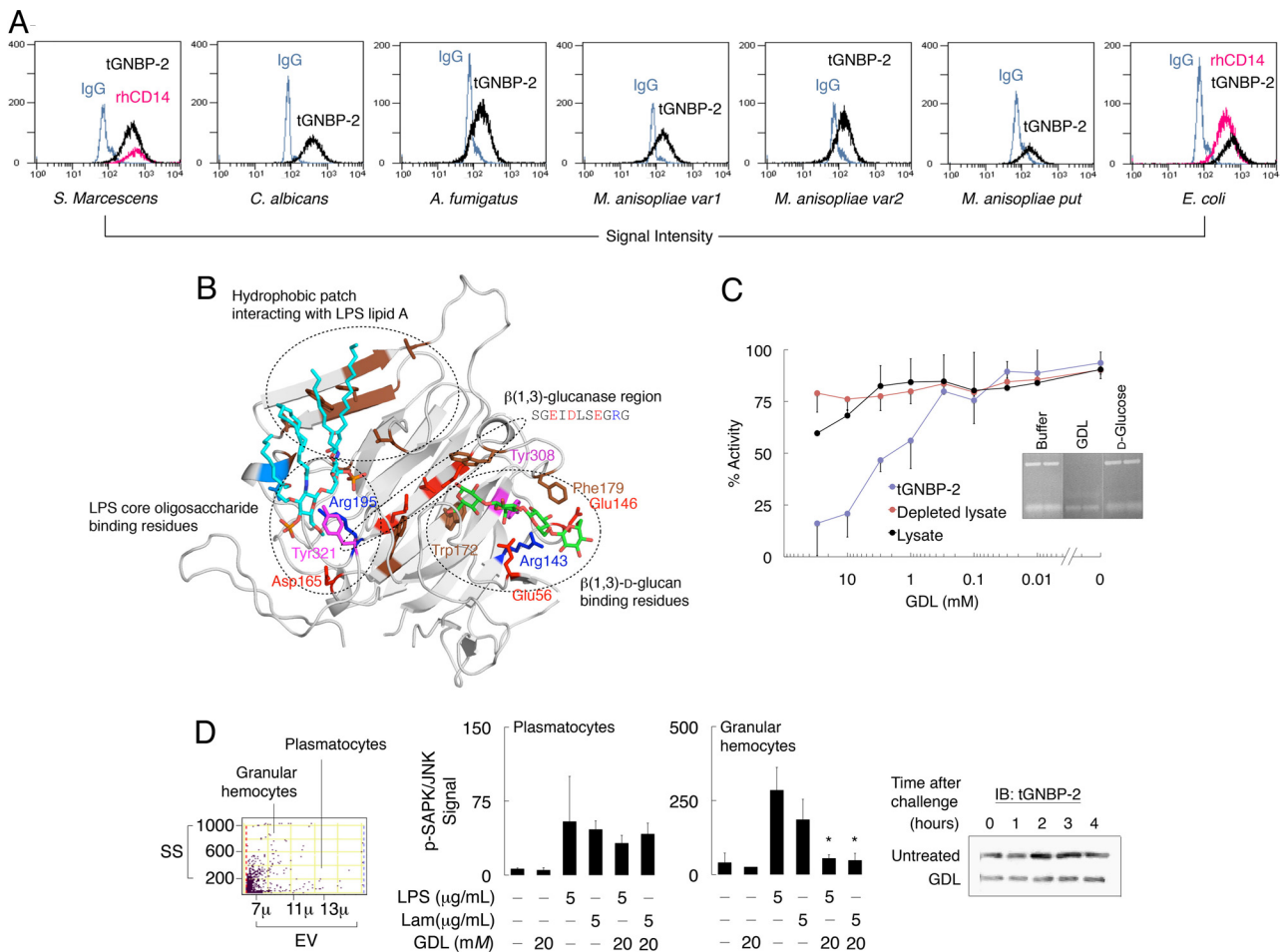
The combination of a pocket, formed by the 6 residues immobilizing the glycan, and the active core that flanks this pocket determines that a  $\beta(1,3)$  glycosidic linkage must be the

reducing-end determinant of the polysaccharide chain and presented to the active core. Therefore, a glucan chain ( $n \geq 1$ ) with a terminal modification was hypothesized to occupy the receptor and hold it an inactive state.

To validate this concept, we started with the simplest chain, a single glucose molecule, whereas a modification of the reducing end would provide a determinant probably sufficient to inactivate the receptor. We therefore selected an existing molecule that completely satisfied these requirements, the glucose derivative D- $\delta$ -gluconolactone (GDL) (29–31).

GDL efficiently blocked the activity of tGNBP-2 but left other  $\beta$ -glucanases intact (Fig. 3C), indicating good specificity for the purpose of this study. In response to a  $\beta(1,3)$ -D-glucan challenge, termite hemocytes responded by stress-activated protein kinase (SAPK)/JNK phosphorylation (32). GDL inhibited this activation response and the subsequent induction of tGNBP-2 (Fig. 3D). Interestingly, however, of the two hemocyte populations expressing tGNBP-2, only granular cells were blocked by GDL,





**Fig. 3.** GDL blocks pattern recognition and signaling by tGNBP-2. (A) Binding of tGNBP-2 to pathogenic bacteria and fungi (*Serratia marcescens*, *Candida albicans*, *Aspergillus fumigatus*, *Metarhizium anisopliae* variants 1 and 2, putative *M. anisopliae* isolated from a dead insect, and *Escherichia coli*) compared with recombinant human CD14 (rhCD14), IgG, isotype controls; tGNBP-2, tGNBP-2 signal; rhCD14, recombinant human CD14 signal. (B) A computational model of tGNBP-2 (gray) with side chains interacting with  $\beta(1,3)$ -D-glucan and eritoran (an LPS analog) colored as follows: red, acidic; brown, hydrophobic; blue, basic; light blue, Asn/Gln; magenta, Tyr.  $\beta(1,3)$ -D-glucan C atoms are green; eritoran C atoms are cyan. In both, O atoms are red, P atoms orange, and N atoms blue. (C) Dose-response inhibition of  $\beta(1,3)$ -glucanase activity of tGNBP-2, termite lysate, or tGNBP-2-depleted lysate by GDL. (Inset) Zymogram of termite extract with GDL or a D-glucose control. (D) Inhibition of SAPK/JNK signaling in hemocytes by suppression of tGNBP-2 measured by flow cytometry (G, granulocytes; M, monocytes; GDL, D- $\delta$ -gluconolactone;  $n = 2,000$ ) (Left). GDL (20 mM) blocks induction of tGNBP-2 by laminarin challenge in termite cells, measured by Western blot (Right).

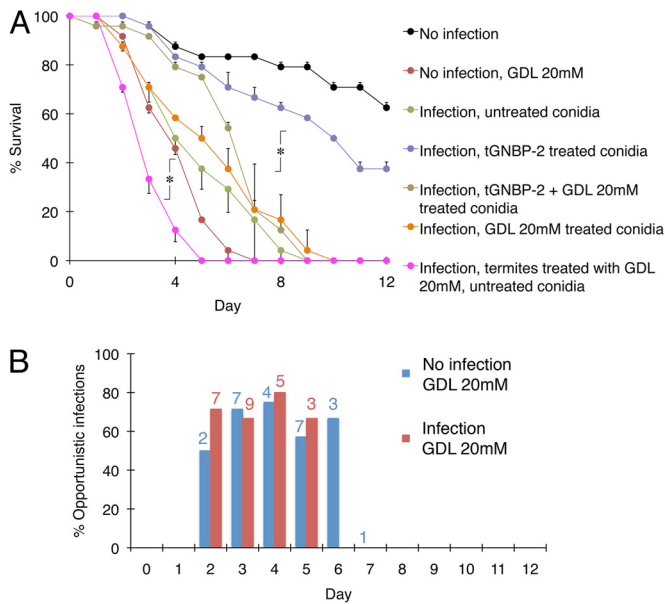
suggesting that plasmotocytes have additional glucan-recognizing mechanisms and might have distinct roles in antimicrobial immunity.

**GDL Suppresses Termite Antimicrobial Immunity In Vivo.** Finally, we aimed at demonstrating the effects of tGNBP-2 blockade on termite survival. Termites died rapidly following infection with *M. anisopliae*. When conidia were treated with isolated tGNBP-2 before infection, termite survival was remarkably similar to that of uninfected groups, indicating that conidia were inactivated by tGNBP-2. However, GDL restored susceptibility to infection. Termites treated with GDL for 24 h before infection showed accelerated mortality (Fig. 4A). GDL had no direct toxic effect on conidia themselves, as any inhibitory effect of fungal glucanases is likely irrelevant before germination. Interestingly, GDL treatment also caused accelerated mortality even in the absence of active infection. This result is explained by postmortem analysis of dead termites from both GDL treatment groups. The analysis showed that termites were killed by *M. anisopliae* as well as by additional opportunistic pathogens, namely, Gram-negative bacteria, such as *Serratia* and *Pseudomonas*, and fungi

(Fig. 4B, Fig. S4, and Fig. S5). Notably, these observations are unlikely to have resulted from inhibited feeding, because termite gut symbionts provide multiple pathways for polysaccharide utilization (33), many of which are not blocked by GDL (34). The different survival kinetics in the in vivo experiments (Figs. 2D and 4A) are due to survivability variations among different *Nasutitermes* colonies used in this study. We are currently investigating potential factors affecting this variability.

### Conclusion

The strategy presented here is an alternative to toxic pesticides given that it is natural, nontoxic, and biodegradable. GDL and similar glycomimetics could potentially be engineered toward a field formulation with minimal adverse effects on surrounding ecosystems, for example, by using nanoparticles to immobilize them. Our strategy may not be limited to termites, because the glucanase activity of tGNBP-2 appears to be shared by several pest species (16–18) as suggested by our sequence and structural analysis. A final interesting note, because GDL is a product of a biosynthetic pathway, plants could conceivably be engineered to produce it in high amounts and at specific compartments,



**Fig. 4.** GDL suppresses termite antimicrobial defense. (A) Effect of gain or loss of tGNBP-2 function on termites infected with *Metarhizium anisopliae* along the course of 12 days (GDL, D- $\delta$ -gluconolactone; mean of 2 groups,  $n = 12$ /group; \*,  $P < 0.05$  tGNBP-2 treated conidia vs. other groups,  $P < 0.05$  GDL treated termites with untreated conidia vs. other groups, n.s. no significant difference vs. no infection). (B) Postmortem analysis of dead termites from GDL treatment groups representing percentage of termites confirmed to have been infected by microbial pathogens. Numbers above bars represent daily death count.

which would bolster their immunity to pest attacks. Glycomimetic synthesis in this context could be optimized as to not interfere with future industrial utilization of plant carbohydrates as a non-fossil-fuel source.

## Materials and Methods

**Study Species.** *N. corniger* nests were collected in April of 2006 from Gamboa in the Republic of Panama. The termites in their carton nests were maintained in plastic boxes at 28 °C and 75% humidity and provided with birch wood and water ad libitum.

**Bioinformatic Analysis.** Insect GNBP s were identified with BLAST search using *Nasutitermes* GNBP s (DQ058898–058922). Representative GNBP s were aligned with ClustalX (35). The phylogenetic tree was constructed by distance analysis with PAUP\* version 4.0b10 (36). The tree was rooted with protein sequence from *B. circulans*  $\beta(1,3)$ -glucanase (AAC60453). One-thousand replicates were used to calculate bootstrap values, and branches were collapsed with a 50% consensus rule.

**Termite Extracts, Tissue Samples, Cuticular Washes, and Hemocytes.** *N. corniger* workers, large workers, or soldiers (whole termites or isolated tissues) were surface sterilized (5% hypochlorite followed by sterile water) and homogenized in acetate buffer (0.2 M, pH 5.0, 4  $\mu$ L per termite) with Biomasher columns (pore size 80–145  $\mu$ m; Cartagen) according to the manufacturer's instructions. Cuticular washes were prepared in 0.1% Tween 80 (10  $\mu$ L per worker). Wash samples were concentrated with a P10 Microcon filter (Millipore). Hemocytes were isolated from 10 chilled *N. corniger* workers (1  $\mu$ L per termite) as described (37).

**Flow Cytometry.** Hemocyte staining was performed essentially as described elsewhere (38). The antibodies used were: primary, anti-tGNBP-2 (5  $\mu$ g/mL) or rabbit IgG (5  $\mu$ g/mL; Sigma); secondary antibody, anti-rabbit PE (1:400; Invitrogen). Intracellular flow cytometry for protein phosphorylation was performed as described (38). The antibodies used were: primary, anti-phospho SAPK/JNK (Fig. S6, 1:100; Cell Signaling); secondary, anti-rabbit fluorescein (1:400; Invitrogen). Flow cytometry was performed on a Beckman-Coulter Cell Lab Quanta SC MPL flow cytometer.

**$\beta(1,3)$ -Glucanase Assays.**  $\beta(1,3)$ -Glucanase activity was measured by a gel electrophoresis assay (39) and by a flow cytometric method (Fig. S7). Briefly, samples were run on Carboxymethyl Curdlan Remazol Brilliant Blue (Loewe Biochemica) gel, and clearing zones representing foci of enzymatic activity were photographed. For flow cytometry, laminarin (Sigma) was labeled with rhodamine green-X succinyl ester (Invitrogen) as described (40) and adsorbed on 3.0- $\mu$ m-diameter polystyrene microspheres (Polysciences) by a 3-h incubation in carbonate/bicarbonate buffer (50 mM, pH 9.6) at 37 °C. Microspheres were then washed and reconstituted in sodium acetate (0.1 M, pH 5.5). Samples were mixed with 1  $\mu$ L of the microsphere suspension, incubated for 15–30 min at 37 °C, and analyzed. Commercially available purified  $\beta(1,3)$ -glucanase from *H. pomatia* or *B. subtilis* was used at 0.1 milliunits/mL as a positive control, and results were expressed as percentage activity of this control.

**Antifungal Assays.** *M. anisopliae* conidia were incubated with single insect extracts (or the equivalent of a single insect from extract prepared from 8–10 pooled workers) in sodium acetate (50 mM, pH 5.0) containing 0.025% Tween 80 and 50  $\mu$ g of ampicillin (40  $\mu$ L of incubation mix included  $\approx$ 100 conidia and crude extract corresponding with one termite). This mix was incubated for 18–24 h at 25 °C, then plated onto potato dextrose agar 100  $\times$  15 mm plates and supplemented with 50  $\mu$ g/mL ampicillin. CFUs were counted following a 4-day incubation at 25 °C. Femtomolar changes in conidial cell volume were directly measured by flow cytometry.

**Size-Exclusion HPLC.** Extract from 30 termites diluted 1:1 in sodium acetate (0.1 M, pH 5.5) was injected into a YMC Pack Diol 200 column (300  $\times$  8 mm ID, particle size 5  $\mu$ m, pore size 20 nm; YMC) and separated on an Agilent 1100 Series instrument at 1 mL/min.

**Immunoprecipitation and Western Blots.** Anti-tGNBP-2 antibodies were raised in rabbits and purified by standard methods (GenScript). Antibodies were covalently linked to 1.0- $\mu$ m-diameter Dynabeads MyOne carboxylic-acid-functionalized magnetic beads (Invitrogen) according to the manufacturer's instructions. Fresh termite extract was diluted 1:1 with immunoprecipitation buffer (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% vol/vol Nonidet P-40, and 1% vol/vol protease inhibitor mixture) and incubated with beads for at least 1 h at room temperature. Beads were washed with immunoprecipitation buffer and eluted with a neutral pH buffer (Pierce). Eluate was desalted and concentrated using a centrifugal column (Vivaspin 10K; Sartorius) and reconstituted in sodium acetate (0.1 M, pH 5.5). SDS/PAGE and Western blots were performed and developed by standard procedures; loading amounts were validated by bicinchoninic acid assay. Anti-tGNBP-2 was used at 2.5  $\mu$ g/mL followed by anti-rabbit HRP (Cell Signaling) at 1:1,000. Soil samples A and B were collected from two different locations in Boston (*Reticulitermes* sp. inhabiting these locations exhibited positive  $\beta(1,3)$ -glucanase activity).

**Homology-Based Structural Modeling of tGNBP-2.** The template structure of an endo- $\beta(1,3)$ -glucanase from alkaliphilic *Nocardioopsis* strain F96 (PDB ID: 2HYK) was chosen by the SWISS-MODEL auto-homology-modeling Web portal to construct a structural model for tGNBP-2. The binding of  $\beta(1,3)$ -glucan was investigated by docking a representative  $\beta(1,3)$ -glucan containing a tetrasaccharide obtained from its cocrystal structure with a *Bacillus macerans* endo- $\beta(1,3)$ -glucanase (PDB ID: 1U0A) to the active site containing the consensus  $\beta(1,3)$ -glucanase sequence. The binding coordinates of eritoran (an LPS analog) were obtained from its cocrystal structure with Toll-like receptor 4/MD2 complex (PDB ID: 2Z65).

**Combinatorial Cytotoxicity Mapping.** Samples of *M. anisopliae* conidia in sodium acetate (0.1 M, pH 5.5) were incubated in 384-well plates with samples from HPLC fractions (see *Size-Exclusion HPLC*) in different combinations (F1 + F1, F1 + F2, F1 + F3, ..., F2 + F2, F2 + F3, ..., F3 + F3, ...; 10  $\mu$ L of each fraction) overnight at 25 °C and analyzed by flow cytometry. tGNBP-2 was depleted from 250  $\mu$ L of each fraction as described in *Immunoprecipitation and Western Blots*.

**In Vitro Binding Assays.** Isolated tGNBP-2 or rhCD14 (R&D Systems) were linked to 3.0- $\mu$ m-diameter polystyrene beads (Polysciences) by a 3-h incubation in boric acid (0.1 M, pH 8.5), followed by 3 washes in cold PBS containing 0.1% vol/vol rabbit serum. Formaldehyde-inactivated pathogenic strains were labeled with FITC as previously described (41). Beads were incubated with labeled pathogens in buffer at 37 °C for 30 min and analyzed by flow cytometry.

**In Vivo Pin-Prick Infection Assay.** Ten workers were cooled on ice and pricked with a sterilized insect pin immersed in a solution of either *M. anisopliae* or *S. marcescens*. Termites were then released at room temperature for specific time intervals of 1.5 and 2.5 h. The experiment was terminated by extracting the termites and freezing the extract for analysis.

**In Vivo Survival and Exposure Assays.** Conidia ( $\approx 3 \times 10^7$ /mL) were centrifuged at  $12,000 \times g$  for 1 min and then suspended in 600  $\mu$ L of 0.1% Tween 80 by vortexing. Two groups of 12 large workers were exposed to filter paper (Whatman 5) moistened with these suspensions in  $35 \times 15$  mm Petri dishes for 20 h. The filter paper was replaced with sterile water-moistened filter paper, and dead termites were removed after a daily census of survivorship. tGNBP-2 treatment was performed with conidia suspended in a sample of the immunoprecipitated protein overnight at 25 °C, followed by extensive washing in PBS containing 0.1% Tween 80. Dead termites were removed daily, surface sterilized with 70% ethanol, and incubated in sterile plates at room temperature for 4 days for postmortem analysis. For exposure to laminarin or LPS,

filter papers were moistened with 300  $\mu$ L of 0.5 or 5 mg/mL solutions of either carbohydrate. Termites were kept on moist filter paper for 24 h before exposure with conidia ( $10^7$ /mL). Controls included termites that were not exposed to sugars, conidia, or both (0.1% Tween 80).

**Statistical Analysis and Significance of Survival Data.** Data were analyzed by Mann–Whitney *U* test and Wilcoxon signed-rank tests with Bonferroni corrections for multiple testing. Termite survival data were analyzed by Cox regression analysis. Data are presented as means  $\pm$  SD.

**ACKNOWLEDGMENTS.** We thank the administrators of the Smithsonian Tropical Research Institute (Republic of Panama) for use of their facilities and obtaining permits for the collection of termite colonies; Prof. R. H. Crozier for helpful comments on this paper; and all of the members of the R.B.R. and R.S. groups for discussions and technical assistance. This research was supported by National Science Foundation Career Development Grant DEB-0447316 (to R.B.R.) and National Institutes of Health Grants GM57073 (to R.S.). I.B. is a Novartis Postdoctoral Fellow of the Life Sciences Research Foundation.

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