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Relative Contributions of Dectin-1 and Complement to Immune Responses to Particulate β-Glucans

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Glucan particles (GPs) are *Saccharomyces cerevisiae* cell walls chemically extracted so they are composed primarily of particulate β-1,3-α-glucans. GPs are recognized by Dectin-1 and are potent complement activators. Mice immunized with Ag-loaded GPs develop robust Ab and CD4+ T cell responses. In this study, we examined the relative contributions of Dectin-1 and complement to GP phagocytosis and Ag-specific responses to immunization with OVA encapsulated in GPs. The in vitro phagocytosis of GPs by bone marrow-derived dendritic cells was facilitated by heat-labile serum component(s) independently of Dectin-1. This enhanced uptake was not seen with serum from complement component 3 knockout (C3−/−) mice and was also inhibited by blocking Abs directed against complement receptor 3. After i.p. injection, percent phagocytosis of GPs by peritoneal macrophages was comparable in wild-type and Dectin-1−/− mice and was not inhibited by the soluble β-glucan antagonist laminarin. In contrast, a much lower percentage of peritoneal macrophages from C3−/− mice phagocytosed GPs, and this percentage was further reduced in the presence of laminarin. Subcutaneous immunization of wild-type, Dectin-1−/−, and C3−/− mice with GP-OVA resulted in similar Ag-specific IgG1 and IgG2a type Ab and CD4+ T cell lymphoproliferative responses. Moreover, while CD4+ Th1 and Th2 responses measured by ELISPOT assay were similar in the three mouse strains, Th17 responses were reduced in C3−/− mice. Thus, although Dectin-1 is necessary for optimal phagocytosis of GPs in the absence of complement, complement dominates when both an intact complement system and Dectin-1 are present. In addition, Th-skewing after GP-basal immunization was altered in C3−/− mice. *The Journal of Immunology*, 2012, 189: 000–000.

β-1,3-α-glucans are homopolymers of glucose that form the structural scaffold of the cell wall of medically important fungi (1). Considering the ubiquitous presence of this fungal pathogen-associated molecular pattern (PAMP), it is not surprising that host defenses have evolved to recognize and respond to β-1,3-α-glucans (2). Dectin-1, a C-type lectin highly expressed on phagocytes, including dendritic cells (DCs), macrophages, and neutrophils, serves as a pattern recognition receptor for β-1,3-α-glucans (3). Upon ligation of β-glucans to Dectin-1, a number of cellular events follow, such as phagocytosis, activation of signaling pathways and transcription factors, generation of reactive oxygen species, and release of cytokines/chemokines (4). The contribution of Dectin-1 to host defenses against fungal infections in vivo has been studied by comparing the susceptibility of wild-type and Dectin-1 knockout mice to fungal challenge. In some but not all models of mycoses, mice with Dectin-1 deficiency manifested increased mortality. For example, Dectin-1 was shown to be required for optimal control of systemic *Candida albicans* infection in one study but dispensable in another study (5, 6). In humans, genetic variations in Dectin-1 and its downstream signaling pathways affect susceptibility to mucosal candidiasis but not candidemia (7, 8).

Particulate β-1,3-α-glucans directly activate the alternative pathway of complement (9–11). iC3b deposited on β-glucans is recognized by complement receptor 3 (CR3; CD11b/CD18) (12). CR3 also has a distinct β-glucan binding site (13). Although complement does not lyse fungi directly, most likely because of the rigid structure of the fungal cell wall, complement plays a unique role in host defenses against opportunistic fungal infections by promoting opsonophagocytosis and by the generation of the potent chemoattractants C3a and C5a (14). Complement has been suggested to play an important role in the immune responses to β-glucans based on studies using β-glucans as an enhancement reagent for mAb-based anti-tumor treatment (15–17).

We have demonstrated that *Saccharomyces cerevisiae*-derived glucan particles (GPs) can be exploited to function as a vaccine adjuvant-delivery platform that elicits strong humoral and Th1- and Th17-biased CD4+ cellular responses to Ags entrapped in the particles (18). GPs are largely composed of β-1,6 branched, β-1,3-α-glucans, with ~2% chitin and less than 1% proteins (19). GPs are recognized by Dectin-1 but are also potent complement activators (10, 18). Thus, in addition to serving as a promising vaccine platform, the size and biochemical properties of GPs make them excellent tools to dissect the role of β-1,3-α-glucans in the immune response to fungi. In this study, we used Dectin-1−/− and C3−/− mice to study the relative contributions of Dectin-1 and complement to the uptake of GPs by mononuclear phagocytes and to immune outcomes after immunization with Ag-loaded GPs.

**Materials and Methods**

**Chemicals and cell culture media**

Chemical reagents were obtained from Sigma-Aldrich (St. Louis, MO), unless otherwise noted. RPMI 1640 medium was purchased from Invitrogen.
Life Technologies (Carlsbad, CA). R10 medium is defined as RPMI 1640 containing 10% FBS (Tissue Culture Biologicals, Tulare, CA), 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM l-glutamine (Invitrogen), and 55 μM 2-mercaptoethanol (Invitrogen). Chicken OVA was purchased from Worthington Biochemical (Lakewood, NJ). Cells were incubated at 37°C in humidified air supplemented with 5% CO2.

**Mice**

Wild-type C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Dectin-1−/− mice were a gift from Gordon D. Brown (University of Aberdeen, Aberdeen, U.K.). C3 mice (The Jackson Laboratory) were bred at the animal facility at the University of Massachusetts Medical School. Both strains of knockout mice were back-crossed at least 12 generations to the C57BL/6J background. Mice were specific pathogen free, and all animal procedures were conducted under a protocol approved by the University of Massachusetts Medical School Institutional Use and Care of Animals Committee.

**Glucan particles**

Glucan particles were prepared from S. cerevisiae (Fleischmann’s Baker’s Yeast) by a series of alkaline and acidic extraction steps as previously described (20–22). Briefly, bone marrow cells obtained from the tibiae and femurs of 8- to 12 wk-old mice were cultured in R10 medium supplemented with 10% GM-CSF conditioned medium from the mouse GM-CSF-secreting J558L cell line. Cells were fed with fresh GM-CSF−supplemented R10 on days 3 and 6. On day 8, nonadherent cells were collected and purified with the Magnetic Cell Separation System (MACS) using CD11c+ magnetic beads (Miltenyi Biotec, Auburn, WA) according to the manufacturer’s protocol. Where indicated, BMDCs were rendered unable to proliferate by incubation in RPMI 1640 containing 50 μg/ml mitomycin C for 30 min at 37°C followed by three washes with R10.

**Mouse bone marrow-derived dendritic cells**

Bone marrow-derived dendritic cells (BMDCs) were generated as previously described with a slight modification (23, 24). Briefly, bone marrow cells obtained from the tibiae of 2- to 3-wk-old mice were cultured in R10 medium supplemented with 10% GM-CSF conditioned medium from the mouse GM-CSF−secreting J558L cell line. Cells were fed with fresh GM-CSF−supplemented R10 on days 3 and 6. On day 8, nonadherent cells were collected and purified with the Magnetic Cell Separation System (MACS) using CD11c+ magnetic beads (Miltenyi Biotec, Auburn, CA) according to the manufacturer’s protocol. Where indicated, BMDCs were rendered unable to proliferate by incubation in RPMI 1640 containing 50 μg/ml mitomycin C for 30 min at 37°C followed by three washes with R10.

**GP opsonization**

Mouse blood was collected by cardiac puncture and immediately transferred in 100 μl dextran (100 mg/ml) or laminarin (100 mg/ml) were injected into the peritoneum of wild-type, Dectin-1−/− or C3−/− mice. After 1 h, 10 ml cold PBS was injected into the peritoneum and collected back. Peritoneal cells were stained with the monocyte/macrophage marker F4/80 (eBioscience) and analyzed by FACS to measure the percentage of F-GP+ cells among F4/80+ cells.

**GP-OVA**

GP-OVA particles were prepared as previously described with slight modification (18, 22). Briefly, 10 mg dry GP-OVA was swollen with 50 μl OVA dissolved in water for 2 h at 4°C to minimize hydrate the GP allowing OVA diffusion into the hollow GP cavity. The samples were then frozen at −80°C and lyophilized. To trap hydraulically the OVA inside the GPs, the dry GP-OVA was again minimally hydrated with water for 2 h at 4°C, frozen at −80°C, and lyophilized. To trap the OVA inside the GPs, the dry GP-OVA formulation were heated to 50°C and 50°C with 50 μl 25 mg/ml tRNA (derived from Torula yeast, type V1, Sigma, St. Louis, MO) in 0.15 M NaCl for 30 min. Then, 500 μl of 5 mg/ml tRNA was added for 1 h at 50°C to complete the complexation reaction trapping the OVA inside the GPs. The GP-OVA particles were washed three times in 0.9% saline, resuspended in 70% ethanol for 30 min, washed three additional times in sterile 0.9% saline, counted, and resuspended at 5 × 105 particles/ml in 0.9% saline. aliquots were stored at −20°C until use. The typical loading efficiency was calculated to be >95% by subtracting the unencapsulated OVA in the supernatant from the initial input using a fluorescent-labeled OVA tracer.

**Immunization**

For immunizations, wild-type, Dectin-1−/−, and C3−/− mice (8–10 wk old, 4 mice per group) received GP-OVA (10 × 105 GPs containing 3.3 μg OVA) in 100 μl s.c. injection over the abdomen. mice were immunized three times at 2-wk intervals and euthanized 2 wk after last immunization. Blood, spleen, and lymph nodes were then harvested for the Ab and T cell assays described in the following sections.

**OVA-specific Ab ELISA**

ELISA was performed as previously described with slight modification (18). Briefly, sera from immunized mice were diluted 1:40 and then progressively diluted 2-fold. Round-bottom 96-well plates coated with OVA were incubated with 50 μl diluted mouse sera at room temperature for 2 h. Wells were then washed and incubated with either biotin-conjugated rat anti-mouse IgG1 (BD Biosciences, San Jose, CA) or biotin-conjugated rat anti-mouse IgG2a (Jackson ImmunoResearch, West Grove, PA) at room temperature for 1 h. After five washes, the plates were incubated for 1 h with streptavidin-conjugated HRP (Roche) and washed five more times, and then developed with tetramethylbenzidine solution (eBioscience). OD450 was measured on a plate reader (Molecular Devices, Sunnyvale, CA). The Ab titer was defined as the dilution factor that was 2-fold greater than the inflection point. Each dilution of serum was tested in duplicate.

**T cell proliferation and ELISPOT assays**

The assays were performed as previously described (18). Briefly, CD4+ T cells were purified from combined lymph node cells and splenocytes using negative selection CD4+ T cell isolation kits (Miltenyi Biotec, Auburn, CA) according to the manufacturer’s protocol. To measure T cell proliferation, purified CD4+ T cells (105/well) were incubated in triplicate or quadruplicate for 4 d with mitomycin C-treated BMDCs (105/well) and the indicated stimuli in round-bottom wells of 96-well plates containing 200 μl R10. Cells were pulsed with [3H]thymidine (1 μCi/well; Perki- nelmer, Boston, MA) for 24 h prior to harvest. Lymphoproliferation was then determined by measuring [3H]thymidine incorporation with a β counter (Wallac 1450 MicroBeta). For the ELISPOT assay, 96-well Multiscreen HTS plates (Millipore, Billerica, MA) were coated with capture mAbs directed at murine IFN-γ, IL-4, or IL-17A. Purified CD4+ T cells (105/well), mitomycin C-treated BMDCs (105/well), and the indicated stimuli were incubated at 37°C for 42–45 h. Samples were run in duplicate except for one experiment that was run singly. Plates were then developed with 5-bromo-4-chloro-3-indolyl phosphate/NBT solution and the number of spots enumerated using an Immunospot ELISPOT reader (Cellular Technology, Shaker Heights, OH).

**Statistics**

Data were analyzed and figures prepared using GraphPad Prism Software. For comparisons of three or more groups, the one-way ANOVA with the
Involvement of CR3 in the ability of serum to facilitate GP phagocytosis

Activation of complement by GPs results in deposition of iC3b on the surface of the particles (10). As CR3 (CD11b/CD18) serves as the major receptor for processed complement component iC3b (12, 13), we next examined the effect of anti-CR3 mAbs on BMDC phagocytosis of GPs. In preliminary studies, we had demonstrated strong expression of CR3 on BMDCs (data not shown). In the presence of fresh wild-type mouse serum, anti-CR3 mAbs had a modest, but significant, inhibitory effect on GP uptake (Fig. 2B). This effect was enhanced when β-glucan receptor binding sites were inhibited with laminarin. These results suggest that CR3 on BMDCs mediates uptake of serum-opsonized GPs. Although CR3 has a direct β-glucan binding site (13), the anti-CR3 mAbs had no effect on uptake of unopsonized GPs or GPs incubated with heat-inactivated mouse serum (Fig. 2A, 2C), suggesting uptake through CR3’s direct β-glucan binding site is minimal.
Roles of Dectin-1 and C3 in mediating GP uptake by peritoneal cells

To study the roles of Dectin-1 and C3 in vivo, we examined the phagocytosis of GPs by peritoneal monocytes/macrophages, as defined by positive staining for F4/80, after intraperitoneal inoculation. For these experiments, some mice were also injected with the soluble β-glucan receptor antagonist laminarin. It has been shown that laminarin does not activate the complement system (10). Dextran, a soluble α-glucan, served as a negative polysaccharide control. Neither dextran nor laminarin had an effect on F-GP phagocytosis in wild-type mice compared with mice containing associated GPs could be found in Dectin-1 knockout mice, regardless of the presence of laminarin (Fig. 3). However, significantly reduced GP uptake by peritoneal monocytes/macrophages was observed in C3−/− mice; this was reduced further by the addition of laminarin (Fig. 3). These results suggest that although both Dectin-1 and complement receptors contribute to recognition of particulate β-glucans in vivo, complement plays a dominant role.

Contributions of Dectin-1 and C3 to immune responses after GP-based vaccination

We have demonstrated that immunization of mice with Ag-loaded GPs elicits both strong humoral and Th1/Th17-biased CD4+ T cell responses (18). To assess the relative contributions of Dectin-1 and complement, anti-OVA Ab development, CD4+ T cell proliferation, and T helper ELISPOT assay were compared in wild-type, Dectin-1−/−, and C3−/− mice, each immunized with GP-OVA. IgG1 and IgG2c anti-OVA Abs were induced at similar titers in all three mouse strains (Fig. 4A, 4B). CD4+ T cells from C3−/− mice proliferated slightly more than those from wild-type mice, both in response to OVA and to the mitogen Con A (Fig. 4C). Although no significant differences were seen in the number of OVA-specific IFN-γ or IL-4-producing CD4+ T cells among the three mouse strains, OVA-specific IL-17A–producing CD4+ T cells from C3 knockout mice were significantly reduced compared with those from wild-type or Dectin-1−/− mice (Fig. 4D).

Discussion

The immunological consequences of ligation of β-glucans with Dectin-1 and other β-glucan receptors have received considerable study in the past decade (reviewed in Ref. 26). However, β-glucans are also potent alternative complement pathway activators (9–11). Thus, to formulate an integrated model of how β-glucans interact with the immune system, the role of complement must be taken into account. In this work, we studied the relative contributions of Dectin-1 and complement to immune responses to GPs. As GPs do not contain mannans, lipids, or proteins, their use allows the dissection of responses to particulate β-glucans without the confounding contributions of these other ligands. We found that complement facilitated the phagocytosis of GPs both in vitro and in vivo. More importantly, Dectin-1 was dispensable for Ab and CD4+ T cell responses to Ag-loaded particulate β-glucans in vivo. In contrast, although complement was redundant for some immune responses, optimal CD4+ Th17 responses required an intact complement system.
We previously demonstrated that Dectin-1 was indispensable for the phagocytosis of GPs by BMDCs in vitro (21). However, those studies used tissue culture media containing heat-inactivated FBS and thus did not take into account that particulate β-1,3,β-glucans are potent complement activators (10). Indeed, when fresh serum was used to opsonize GPs, phagocytosis by BMDCs proceeded, even if Dectin-1–deficient BMDCs were used or if β-glucan receptors were blocked with laminarin. Opossonization of GPs with complement-sufficient serum results in dispersal on the GP surface of iC3b (10). Whereas some DC populations do not express the iC3b receptor, CR3, BMDCs do, and blocking of CR3 with anti-CR3 mAbs inhibited the uptake of opsonized GPs. This effect was more profound when β-glucan receptors were blocked with laminarin. However, some phagocytosis was still observed that could have been mediated by the DC cell surface protein, CD11c/CD18, which also binds iC3b (27).

In some experimental systems, Dectin-1 is required for β-glucan–induced Th17 development in vitro (28–30). In addition, recent in vivo studies show that Dectin-1 is essential for the adjuvant effect of the bacterial β-1,3-β-glucan, curdlan, and the anti-tumor activity of particulate β-glucans (30, 31). However, in our studies, wild-type and Dectin-1−/− mice immunized with GP-OVA developed similar OVA-specific Ab titers, CD4+ T cell proliferative responses, and CD4+ T cell skewing. The reason for this discrepancy is speculative but may be explained by differences in how the Ags were delivered. In our system, OVA was encapsulated inside of GPs and thus the Ag and β-glucan were targeted directly to the same compartments within APCs. In contrast, in the aforementioned studies, the Ags and β-glucans were admixed and therefore would not necessarily traffic to the same intracellular compartment. Colocalization of Ag and PAMPs has been shown to increase the efficiency of the T cell response (32) and may thereby diminish the need for Dectin-1. In addition, in the absence of Dectin-1, complement receptors and other β-glucan receptors, such as the scavenger receptors SCARF1, CD36, and CD5, may compensate for the activities of Dectin-1 (33–35). Indeed, β-glucan signaling through CD36 has been shown to induce cytokines (34).

Although the complement system was originally thought to contribute only to innate immunity against pathogens, more recent studies support the concept that complement activation can instruct B and T cell development (reviewed in Refs. 36, 37). Complement activation by GPs results in the generation of the cleavage products C3a and C5a (10). Interaction of these chemotraffacts with their cognate receptors may influence Th17 development. In models of asthma and infection, mouse strains deficient in C3 or C5 have impaired CD4+ Th17 cell responses, whereas C5a receptor knockout mice mount enhanced IL-17A responses (38–40). The importance of the C5a–C5aR pathway in Th17 cell development also has been demonstrated in an autoimmune arthritis model (41). C3 is central to all three complement activation pathways, and C3 deficiency will generally result in the loss of complement function. Notably, however, in the absence of C3, thrombin can substitute as a C5 convertase to generate C5a, albeit at reduced levels (42). Future studies will be needed to dissect the contributions of C3a and C5a to the Th17 responses observed after immunization with Ag-loaded GPs. Additionally, the relative contributions of the alternative and classical pathways of complement activation needs delineation. Although GPs directly activate the alternative pathway (10), recent data demonstrating that mice have natural IgM Abs against β-glucans suggest that the classical complement pathway could also be activated by GPs (43).

How the immune system specifically responds to particulate β-glucans is difficult to reconcile with models that use live fungal challenges. In addition to β-glucans, fungi have multiple other PAMPs, such as mannans, that are innately recognized by the host immune system (2). In addition to serving as a vaccine platform, Ag-loaded GPs serve as a tool to study how β-glucan recognition contributes to immune responses to fungal Ags. Our data suggest a redundant role for Dectin-1 in the generation of systemic CD4+ T cell and Ab responses to Ags within particulate β-glucans. This redundancy appears to be explained by the ability of complement activation by β-glucans to license immune responses to associated Ags. However, it will be important to examine whether Dectin-1 is required for optimal mucosal immune responses, particularly given the association of mutations in Dectin-1 genes with mucosal candidiasis (7). In summary, our data suggest a dominant role for complement and a redundant role for Dectin-1 in both phagocytosis of GPs and CD4+ Th17-skewing after GP-based immunization.

Disclosures

The authors have no financial conflicts of interest.

References


