ß-glucan affects leukocyte navigation in a complex chemotactic gradient

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Background. *Polymorphonuclear leukocytes (PMNs) must traverse endogenous chemotactic gradients (interleukin 8 [IL-8]) before reaching target chemoattractants (fMLP [N-formylmethionine-leucine-phenylalanine], C5a) produced at a site of bacterial infection. Complement receptor 3 (CR3; CD11b/CD18) contains 2 distinct binding sites, one that mediates adhesion and a lectin-like domain (LLD) that binds polysaccharides of microbial origin. This laboratory previously reported an increase in the chemotactic capacity of PMNs toward fMLP upon ligation of the CR3 LLD with β-glucan, a CR3 agonist. Current studies sought to determine the effect of β-glucan on PMN navigation toward other chemoattractants alone and in a competing chemotactic environment.*

Methods. *Migration was assessed by serum agarose overlay with the use of chambered slides containing or not, β-glucan. Migration of human PMNs at 37°C for 2 hours was evaluated toward C5a or IL-8 alone and in competing gradients. Selected groups were treated with anti-CR3—blocking antibodies. The number of chemotactic cells was quantified by microscopy.*

Results. *β-glucan significantly enhanced chemotaxis toward C5a and suppressed that toward IL-8 in a CR3-dependent fashion. In the competing chemotactic gradient assays (C5a vs IL-8), β-glucan further enhanced migration toward C5a while not affecting that toward IL-8.*

Conclusions. *β-glucan selectively upregulates PMN chemotaxis toward C5a while suppressing chemotaxis toward IL-8. (Surgery 2004;136:384-9.)*

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AFTER EXTRAVASATION, NEUTROPHILS (PMNs) interpret a multitude of chemotactic gradients en route to an infectious source or site of injury. Neutrophils migrate toward infected tissues initially through gradients of host-derived chemoattractants such as interleukin-8 (IL-8) and leukotriene B4 (LTB4) that are secreted by nearby macrophages, mast cells, and other myeloid cells.[1] PMNs then follow gradients of bacterial products (fMLP [N-formyl-methionine-leucine-phenylalanine]) and complement fragments (C5a), which are produced at the site of infection and serve as end-target chemoattractants.[2] It has been proposed that, despite the presence of high concentrations of host-derived chemokines, preferential neutrophil migration toward end-target chemoattractants demonstrates the existence of a chemoattractant hierarchy with some molecules being dominant over others.[3]

Neutrophil migration is mediated by integrins, heterodimeric molecules that regulate cell—cell and cell—extracellular matrix interactions.[4] Complement receptor 3 (CR3; CD11b/CD18) is a β_2 integrin necessary for optimal migration of leukocytes into sites of inflammation or infection.[5] CR3 plays a role in neutrophil adhesion and transmigration through an endothelial cell layer and in migration through the extracellular matrix. CR3 can bind an extensive repertoire of ligands, which renders this integrin able to mediate a number of functions in host defense. A region within the extracellular domain of CR3, referred to as the inserted or I-domain, has been shown to bind more than 30 ligands including a number of extracellular matrix proteins such as fibrinogen, fibronectin, laminin, collagen, vitronectin, and thrombospondin.[6]

In addition to the I-domain, CR3 contains a unique lectin-like domain (LLD) that permits binding of microbial polysaccharides such as β1,3-linked glucose polymers (β-glucan).[7,8] ß-glucans are structural polymers of the fungal cell wall that prime leukocyte function due, in part, to their ability to ligate the LLD of CR3.[9] Dual ligation of

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the I-domain and the LLD by their corresponding ligands has been shown to affect CR3-dependent functions differently than ligation of either site alone. Co-occupancy of the I-domain with the extracellular matrix protein fibronectin, and of the LLD with β-glucan, resulted in an increase in the chemotactic capacity of neutrophils toward fMLP.[10] The present study was performed to determine whether the CR3-dependent effect of ß-glucan extends to PMN chemotaxis toward other chemoattractants presented in singular as well as in complex gradients. Findings in this report demonstrate that ß-glucan alters the migratory behavior of neutrophils toward C5a and IL-8 in a diametrically opposing fashion.

MATERIAL AND METHODS

Reagents. Highly purified, pharmaceutical grade, soluble β-glucan (BETAFECTIN, 150,000± 20,000 mw) was obtained from Biopolymer Engineering Inc (Eagan, Minn). Dextran was purchased from Sigma (St. Louis, Mo). Phosphate-buffered saline (PBS) and Hanks' balanced salt solution (HBSS) were obtained from Gibco Life Technologies (Grand Island, NY). RPMI medium 1640 was purchased from Invitrogen Corp (Grand Island, NY) and fetal bovine serum (FBS) from Hyclone Labs (Logan, Utah). Agarose was obtained from Seakem GTG, FMC Bioproducts (Rockland, Me). The chemoattractants C5a and IL-8 were purchased from Calbiochem (La Jolla, Calif). All reagents used contained less than 0.1 pg endotoxin, as determined by Limulus amoebocyte lysate screening (Biowhittaker, Walkersville, Md). The CD11b-specific antibody VIM12 was purchased from Caltag Laboratories (Burlingame, Calif). The CR3 I-domain−specific antibody LM2.1 was obtained from Bender Medical Systems (San Bruno, Calif). Human HLA Class I antibody was purchased from Sigma.

Isolation of human neutrophils. Human neutrophils were isolated from the peripheral venous blood of healthy volunteers in heparinized Vacutainer tubes (Becton Dickinson, Lincoln Park, NJ). Granulocytes were prepared by gradient centrifugation on Ficoll-Hypaque (Sigma), followed by erythrocyte sedimentation with 3% dextran (500,000 mw). The leukocyte-rich supernatant underwent hypotonic lysis of residual erythrocytes. Cells were resuspended in ice-cold HBSS and counted. PMN purity and viability were consistently greater than 95%.

Antibody treatment of human neutrophils. Neutrophils (3×10^6 cells/mL), were incubated with no antibody, LM2.1 (5 μg/10^6 cells), VIM12 (5 μg/10^6 cells), or anti-HLA class I immunoglobulin G (IgG; 5 μg/10^6 cells) for 30 minutes on ice. Anti-HLA class I IgG was used to control nonspecific effects of antibody binding to cell-surface antigens. After antibody treatment, neutrophils were washed with sterile PBS and added onto the migration assay.

Slide preparation. Two-well, chambered slides (Lab-Tek Permanox Chambered Slides; Fisher Scientific, Fair Lawn, NJ) were coated with 1 mL soluble ß-glucan (100 μg/mL, unless indicated otherwise) for 30 minutes at 37°C in 5% CO_2. Before use, wells were washed with PBS and allowed to air dry.

Migration assays. The underagarose assay was performed as described.[2] Each well of a 2-well, chambered slide was filled with 2 mL of a 0.5% agarose solution containing 50% H_2CO_3-buffered HBSS and 50% RPMI-1640 culture medium with 20% heat-inactivated FBS. The agarose solution in the β-glucan pretreated wells also contained β-glucan at a final concentration of 100 μg/mL or as otherwise indicated. After the agarose solidified, the slides were allowed to equilibrate for 1 hour at 37°C with 5% CO_2. Using a plastic template and beveled punch, we created three 2-mm wells, each separated by a distance of 2 mm. The agarose plugs were removed with gentle aspiration. When a single chemoattractant assay was undertaken, 10 microliters containing 10 pmols of either C5a or IL-8 were placed in the central well. The outer wells each received 1×10^6 cells resuspended in 10 μL of HBSS. The slides were incubated for 2 hours at 37°C with 5% CO_2 and then fixed with 3.7% neutral buffered formalin. When a competing chemoattractant assay was undertaken with C5a and IL-8 gradients, 1 outer well was loaded with C5a, the second outer well was loaded with IL-8, and the middle well with neutrophils. Directional migration of the cells was quantified with the use of an Olympus CK2 microscope. To facilitate cell counting, we aligned a grid with the edge of the cell-containing well facing the chemoattractant. To ensure that we excluded randomly migrating cells and that we accounted for only chemotactic cells, we counted only the cells that had migrated beyond the second row of the grid in the exact manner described in Heit et al.

Statistical analysis. Statistical assessments were made with the Student *t* test or ANOVA with post hoc analysis; significance was set at $P < .05$.

RESULTS

ß-glucan promotes neutrophil migration toward C5a and decreases migration toward IL-8. An initial series of experiments showed that ß-glucan

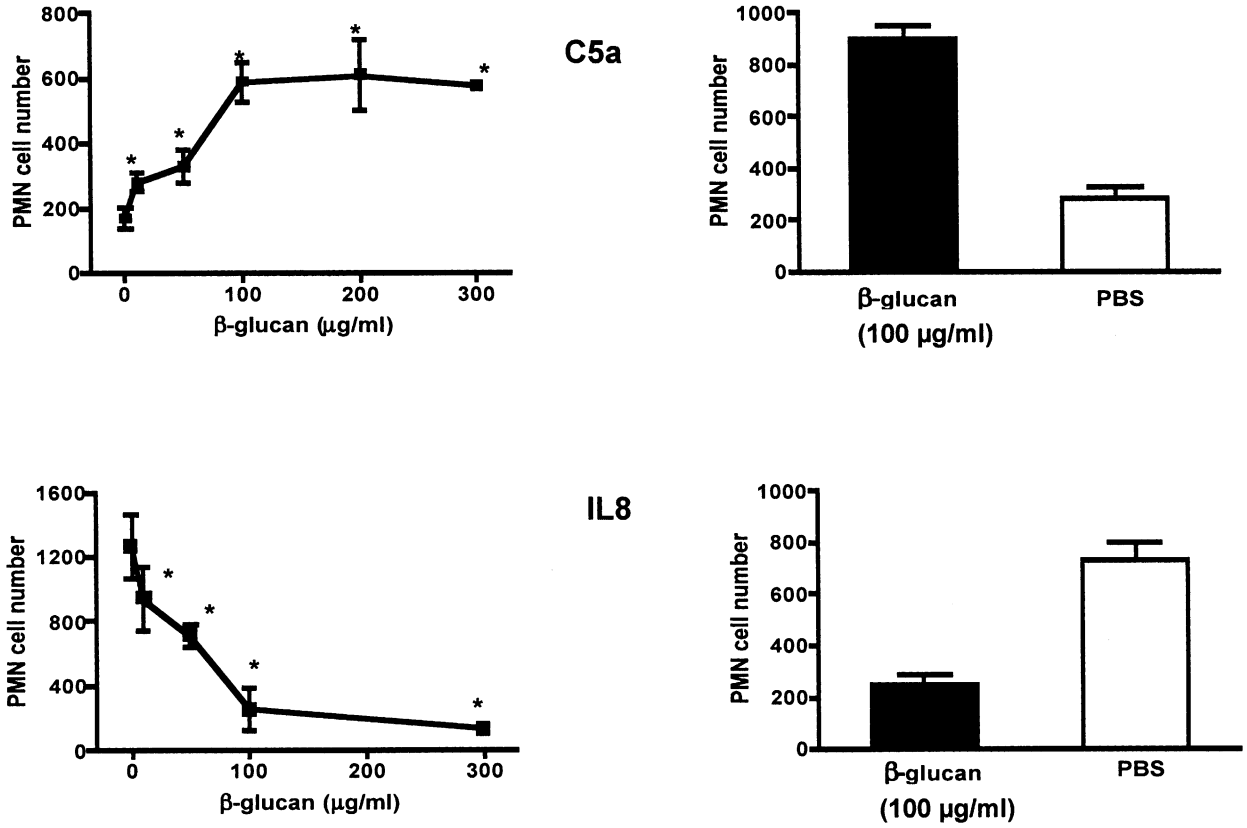

Fig 1. Differential effect of ß-glucan on neutrophil chemotaxis. Migration assays were performed as described in Material and Methods. **A,** ß-glucan (0-300 μg/mL) induces a dose-responsive increase in the number of PMNs migrating toward C5a (10 pmols). Data shown are mean ± SD of 4 separate wells. *$P < .05$ vs no ß-glucan. **B,** PMN migration toward C5a (10 pmols) in the presence and absence of ß-glucan (100 μg/mL). Data shown are mean ± SEM of 6 separate experiments. $P < .01$ (*t* test). **C,** ß-glucan (0-300 μg/mL) induces a dose-responsive suppression of PMN migration toward IL-8 chemoattractant (10 pmols). Data shown are mean ± SD. *$P < .05$ vs no ß-glucan. **D,** PMN migration toward IL-8 (10 pmols) with and without ß-glucan (100 μg/mL). Data shown represent mean ± SEM of 6 separate experiments; $P < .01$ (*t* test).

induced a dose-responsive promotion of PMN migration toward C5a with a maximal increase in chemotaxis seen at agarose concentrations of 100 μg/mL ß-glucan (Fig 1, *A*). Figure 1, *B* shows the results of 6 independent experiments in which 100 μg/mL ß-glucan was found to cause a 3-fold increase in the number of PMNs migrating toward a C5a chemotactic gradient over a 2-hour time course ($P < .01$ vs control). The concentration of C5a (10 pmols) was determined to be optimal for chemotaxis in the presence of 100 μg/mL ß-glucan (data not shown).

In contrast to the promotion of neutrophil chemotaxis induced by ß-glucan toward a C5a gradient, ß-glucan caused a dose-responsive inhibition of migration toward IL-8 (Fig 1, *C*). As for C5a, 100 μg/mL ß-glucan caused a maximal effect on neutrophil chemotaxis and was used in subsequent experiments. Figure 1, *D*, shows the results of 6 independent experiments in which a 3-fold reduction in the number of cells migrating toward a gradient established with 10 pmols IL-8 was found in the presence of ß-glucan ($P < .01$).

Neutrophil migration is CR3 dependent. Previous results from this laboratory showed that ß-glucan enhanced neutrophil chemotaxis toward fMLP in a CR3-dependent mechanism.[10] In the present study, PMN migration was assessed in the presence or absence of the monoclonal antibody LM2.1, which blocks CR3 function by binding to the I-domain of CR3. Results presented in Fig 2 show that LM2.1 significantly inhibited migration toward C5a, regardless of the presence of ß-glucan. Similar inhibition of chemotaxis toward IL-8 was found (not shown), demonstrating that the integrin CR3 is important in neutrophil chemotaxis

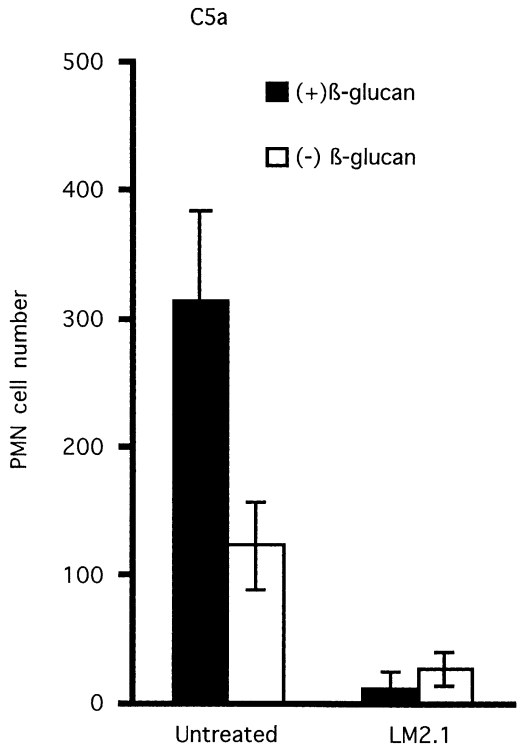

Fig 2. Inhibition of PMN chemotaxis by anti-CR3 I-domain–specific mAb LM2.1. PMNs were pretreated with LM2.1, or not, as described in Material and Methods. The number of migrating cells toward C5a (10 pmols) determined after 2 hours in the presence or absence of ß-glucan (100 μg/mL). Data represent mean ± SEM of 3 separate experiments; *P < .01 vs no ß-glucan (t test).

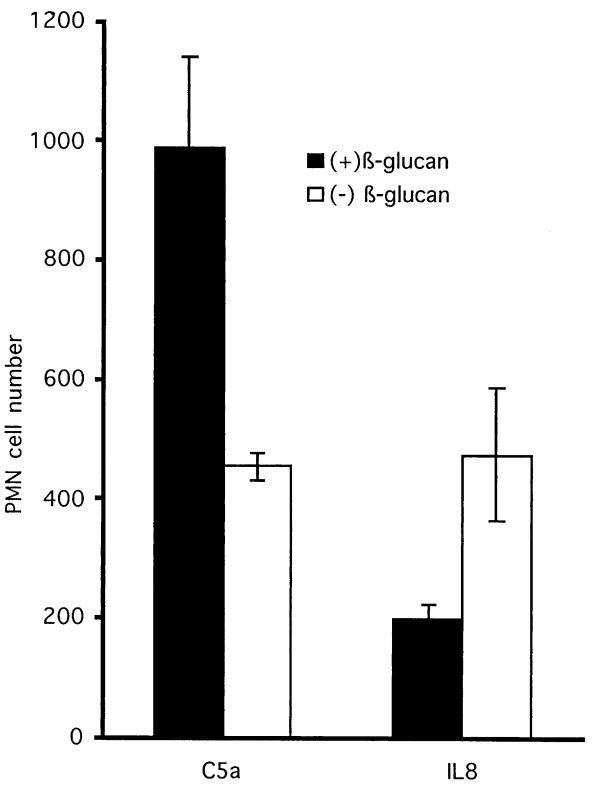

Fig 3. Migration of neutrophils in competing chemotactic gradients of C5a and IL-8. C5a (10 pmols) and IL-8 (10 pmols) were placed in the 2 outer wells, and PMNs were placed in the central well of an underagarose assay with, or without, ß-glucan suspended in the agarose at 100 ug/mL. Results are shown as mean ± SEM of 6 separate experiments; *P < .05 vs no ß-glucan.

toward both C5a and IL8 chemoattractants, regardless of the presence of ß-glucan.

ß-glucan regulates PMN migration in a complex chemotactic environment. The differential effects of ß-glucan on neutrophil migration toward C5a and IL-8 was tested under conditions in which both chemoattractants were presented to neutrophils at the same time. The rationale for this experiment is found in recent reports showing that the presence of IL-8 in an opposing gradient significantly affected PMN migration toward either fMLP or C5a, resulting in an increase in the number of migrating cells to these end-target chemoattractants.[2] In this context, observations regarding PMN migration toward a single chemoattractant should be confirmed in the more physiologically relevant setting wherein PMNs encounter both host- and bacterial-derived chemoattractants. We therefore determined whether the promoting effect of ß-glucan toward C5a would be obfuscated by the presence of IL-8. In opposing gradients of C5a and IL8, neutrophils migrate preferentially toward the end-target chemoattractant C5a (Fig 3). The differential effect of ß-glucan on PMN chemotaxis was maintained in an opposing gradient, promoting migration toward C5a and inhibiting migration toward IL-8 (Fig 3).

Antibody blockade of the CR3 LLD obviates the effect of β-glucan on neutrophil chemotaxis. To test the hypothesis that the effect of ß-glucan on neutrophil migration is mediated by the LLD of CR3, we performed antibody-blocking experiments as described in Material and Methods. VIM12 is a monoclonal antibody that binds to the LLD of CR3; the anti-HLA class I IgG monoclonal antibody was used to control for nonspecific effects of antibody binding to cell-surface antigens. Results in Fig 4 show that, in the presence of ß-glucan, VIM12-treated neutrophils no longer showed enhanced chemotaxis toward C5a and demonstrated normal levels of migration toward IL-8, indicating that the effect of ß-glucan on neutrophil chemotaxis is mediated by the LLD of CR3. The anti-HLA

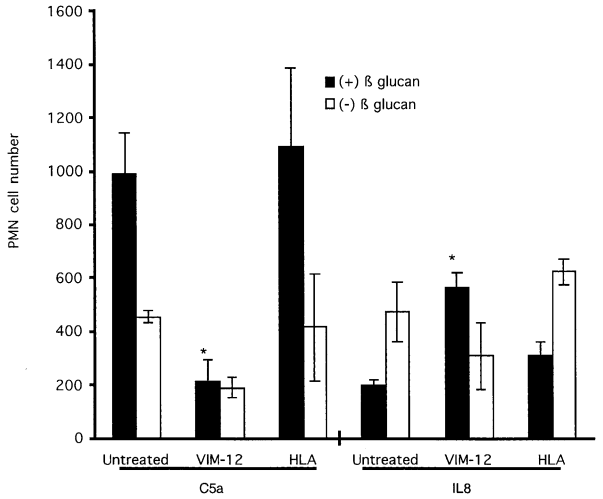

Fig 4. Antibody to CR3 LLD blocks ß-glucan effect on PMN chemotaxis. PMNs were pretreated with VIM12 (anti-LLD) mAb as described in Material and Methods, and placed in the central well of a competing chemotactic gradient of C5a (10 pmols) and IL-8 (10 pmols). Class I HLA IgG served as the cell-surface control mAb. Data are mean ± SEM of 6 separate experiments; $*P < .05$ compared with migration of untreated PMNs.

monoclonal antibody did not alter the effect of ß-glucan on either chemoattractant.

DISCUSSION

CR3 is unique among leukocyte integrins in that it possesses a lectin-like binding site in addition to the site responsible for mediating its adhesive function.[11] Whereas the adhesive function of CR3 has been widely studied, the effect of ligation of the lectin site on neutrophil function is not well understood, although a number of studies have supported its potential for enhancing innate immune function. For example, ß-glucan, a natural ligand of LLD, has recently been shown to enhance the ability of antitumor mAbs to cause tumor regression and increase survival in mice by a mechanism dependent on leukocyte CR3.[12] Previous studies from this laboratory showed that ß-glucan promoted the migration of neutrophils toward the bacterial-derived, end-target chemoattractant fMLP and did so via a mechanism dependent on binding to the CR3 LLD.[10] In a pathophysiologic setting, neutrophils navigate through gradients of multiple chemoattractants en route to a site of infection.[3] This investigation shows that ß-glucan upregulates neutrophil migration toward the end-target chemoattractant C5a, while it downregulates migration toward the host-derived chemoattractant IL-8. Further, in a competing chemotactic environment with C5a and IL-8 present, the enhanced migration toward C5a is retained. These findings extend the known biological consequences of ß-glucan on neutrophil chemotaxis by showing that its effect is dependent on the nature of the chemoattractant.

Antibody-blocking studies (Fig 2) demonstrate that neutrophil migration to both C5a and IL-8 is CR3 mediated, since treatment of the cells with the CR3 I-domain–specific mAb LM2.1 significantly inhibited migration to both chemoattractants. Furthermore, site-specific blocking of the CR3 LLD with mAb VIM12 (Fig 4) obviated the ß-glucan effect on neutrophil chemotaxis in a competing chemotactic environment with both C5a and IL-8 present, thus demonstrating that the CR3 LLD can regulate neutrophil chemotaxis when ligated with an agonist such as ß-glucan. Previous studies have shown that ß-glucan enhances PMN migration toward fMLP through the differential regulation of ß1 and ß2 integrins.[10] The mechanism through which ß-glucan enhances chemotaxis toward C5a and restricts migration toward IL-8 is not yet known.

As mentioned, our data reveal that the ligation of the CR3 LLD has opposing effects on neutrophil migration, enhancing migration toward end-target chemoattractants (fMLP, C5a) while suppressing chemotaxis toward the host-derived chemoattractant IL-8. Reports by others have shown that host-derived and end-target chemoattractants mediate PMN migration via distinct intracellular signaling pathways. The p38 MAPK pathway plays an important role in PMN migration to end-target chemoattractants, whereas phosphatidylinositol-3 kinase (PI3K) mediates chemotaxis toward host-derived chemoattractants.[13-15] The ß-glucan studies presented here directly support the findings from the second messenger pathway studies by demonstrating, through different means, that chemoattractants exert distinct intracellular effects despite the fact that they all bind to a common class of G-coupled protein receptors.[13] Ongoing work will test the hypothesis that ß-glucan preferentially primes the P38 MAPK pathway while it downregulates PI3K signaling, an outcome which could account for its enhancement of migration toward C5a and decreased migration toward IL-8, respectively.

The nature of the ß-glucan receptor has been of great interest over the years; several strong candidates, in addition to the $ß_2$ integrin CR3, have been reported.[9,16] Dectin-1, a recently described ß-glucan receptor, is the dominant receptor on macrophages that mediates phagocytosis of yeast, whereas CR3 is the predominant receptor on

neutrophils.[17] In further support of the predominant role of CR3 in mediating neutrophil responsiveness to ß-glucan, the present study shows ß-glucan regulation of neutrophil chemotaxis in chemotactic gradients with C5a and/or IL-8 to be fully accountable by its binding to the LLD of CR3.

The ß-glucan used in our currently reported studies is unique among known biological response modifiers with immune-enhancing properties. This is so because ß-glucan demonstrates the ideal clinical quality in that it increases the antimicrobial function of human leukocytes but does not elicit proinflammatory cytokine production and was therefore well tolerated in clinical trials.[18] Whether suppressed migration of neutrophils toward host-derived chemokines with concomitant increase toward bacterial-derived chemoattractants is a desirable property of an immune-enhancing agent remains to be seen. One may speculate that this selective effect on chemotaxis allows neutrophils to more readily transit proximal chemotactic gradients while more efficiently responding to chemoattractants emanating from sites of infection. Taken together, these cells might be more effective in navigating through the multiple chemotactic signals encountered in an infected or injured tissue and would arrive at the end-target site with greater expediency.

CONCLUSION

This report demonstrates that the CR3 LLD can selectively upregulate leukocyte migration toward the end-target chemoattractant C5a while decreasing migration toward the host-derived chemokine IL-8, further establishing a hierarchical route of neutrophil navigation in a complex chemotactic environment.

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