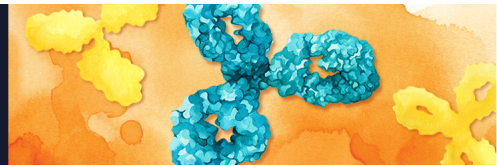


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Lauren A. McVoy and Richard R. Kew

J Immunol 2005; 175:4754-4760; ;
doi: 10.4049/jimmunol.175.7.4754
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The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.



CD44 and Annexin A2 Mediate the C5a Chemotactic Cofactor Function of the Vitamin D Binding Protein¹

Lauren A. McVoy and Richard R. Kew²

The vitamin D binding protein (DBP) is a plasma protein that significantly enhances the chemotactic activity of C5a and C5a^{desArg} (cochemotactic activity). The objective of this study was to investigate how DBP mediates this process using neutrophils and U937 cells transfected with the C5a receptor (U937-C5aR cells) and comparing chemotaxis to C-activated serum (DBP dependent) vs purified C5a (DBP independent). Binding to the cell surface is essential for this protein to function as a chemotactic cofactor, and DBP binds to a chondroitin sulfate proteoglycan (CSPG) on neutrophil plasma membrane preparations. To determine whether a CSPG also functions to mediate cochemotactic activity, U937-C5aR cells were grown in chlorate to inhibit CSPG sulfation or treated with chondroitinase AC. Either treatment significantly inhibited chemotaxis only to C-activated serum. CD44 is a major cell surface CSPG on leukocytes, and functions to facilitate chemotaxis. Treatment of cells with anti-CD44 blocks chemotaxis of neutrophils and U937-C5aR cells to C-activated serum but not purified C5a. DBP binds to CD44 on the cell surface as evidenced by coimmunoprecipitation, confocal microscopy, and cell binding studies. Annexin A2 associates with CD44 in lipid rafts; therefore, its potential role in mediating cochemotactic activity was investigated. Results demonstrate that anti-A2 inhibits neutrophil and U937-C5aR chemotaxis specifically to C-activated serum, blocks DBP binding to cells, and colocalizes with anti-DBP on the cell surface. These results provide clear evidence that CD44 and annexin A2 mediate the C5a chemotactic cofactor function of DBP. *The Journal of Immunology*, 2005, 175: 4754–4760.

The chemotactic activity of C activation peptides C5a and C5a^{desArg} (the stable serum form) can be enhanced significantly by the vitamin D binding protein (DBP),³ a plasma protein also known as Gc-globulin (1–8). This positive chemotactic cofactor function of DBP (i.e., cochemotactic activity) is specific for the C5-derived peptides and, moreover, it cannot augment other C5a-mediated leukocyte functions (oxidant generation and degranulation) (1–8). DBP is an abundant multifunctional 56-kDa plasma protein that is part of the albumin gene family (9). Although the protein by itself lacks chemotactic activity, it associates with the plasma membrane of many cell types and appears to bind with low affinity to multiple cell surface ligands such as chondroitin sulfate proteoglycans (CSPGs) (10), megalin (11), and cubulin (12). Neutrophils transiently generate cochemotactic activity for C5a/C5a^{desArg} on the cell surface within 15–20 min of DBP binding (13). These cells also use membrane-bound elastase to shed the DBP binding site into the extracellular milieu (14). Both plasma membrane binding and subsequent shedding of DBP are essential for the protein to function as a chemotactic cofactor for C5a.

The DBP binding site complex has been inferred by functional, structural, and kinetic cell binding studies (10, 13–15). Recently, we have speculated that formation of a DBP binding site complex is a dynamic, multistep, and transient process involving perhaps several distinct cell surface macromolecules (15). However, the precise components that form the binding site complex as well as the mechanism(s) by which DBP enhances chemotaxis to the C5-derived peptides are not known. Therefore, the objective of this study was to identify cell surface molecules that may mediate the C5a chemotactic cofactor function of DBP. For this study, both peripheral blood neutrophils and the U937 cell line stably transfected with the C5a receptor (U937-C5aR) were used (16). U937-C5aR cells express similar levels of cell surface C5a receptors as do mature peripheral blood neutrophils and display a chemotactic dose-response curve to purified C5a (and C5a^{desArg}) that is identical to neutrophils (16). In contrast to neutrophils, U937-C5aR cells respond only to C5a or C5a^{desArg}. Previously, using neutrophils (1) and U937-C5aR cells (15), we have demonstrated unequivocally that the chemotactic activity in C-activated serum (activated using zymosan) is due to a combination of C5a^{desArg} plus DBP. Immunodepletion of DBP reduces the chemotactic activity in C-activated serum by almost 75% without altering the levels of C5a^{desArg} (15). Therefore, the relative contribution of a particular cell surface molecule in mediating the cochemotactic effect of DBP can be determined using specific Abs or inhibitors to block U937-C5aR movement to C-activated serum. Using this approach, the results clearly demonstrate that the transmembrane glycoprotein CD44 (a CSPG) and annexin A2 (cell membrane Ca²⁺/phospholipid binding protein) both function to mediate the chemotactic cofactor function of DBP.

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Received for publication March 29, 2005. Accepted for publication July 19, 2005.

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¹ This investigation was supported in part by a grant to R.R.K. from the National Institutes of Health (GM 63769). L.M. was supported in part by a Medical Scientist Training Program Grant from the National Institutes of Health. This work has been submitted by L.A.M. in partial fulfillment of the requirements for the Ph.D. program in Molecular and Cellular Biology, Stony Brook University.

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³ Abbreviations used in this paper: DBP, vitamin D binding protein; CSPG, chondroitin sulfate proteoglycan.

Materials and Methods

Reagents

Purified recombinant human C5a was purchased from Sigma-Aldrich. DBP was purified from human plasma and purchased from Biotest International. BSA, goat IgG, ionomycin, DNase I, chondroitinase AC, and zymosan A (yeast cell walls from *Saccharomyces cerevisiae*) were obtained

from Sigma-Aldrich. The protease inhibitors PMSF and 1,10-phenanthroline were purchased from Sigma-Aldrich, whereas Pefabloc SC and E-64 were purchased from Roche Applied Science. Polyclonal Abs to CD44 and annexin A2, directed against a specific peptide epitope, were produced in goats. The affinity-purified Abs, CD44 (N-18) and annexin A2 (C-16) and their corresponding peptide Ags (Ag blocking peptide) were obtained from Santa Cruz Biotechnology. Polyclonal anti-DBP was purchased from DiaSorin and then affinity-purified using immobilized DBP. Monoclonal anti-DBP (MAK-89) was a generous gift from the late Dr. J. Haddad (University of Pennsylvania, Philadelphia, PA). Sterile, pyrogen-free water, HBSS, PBS, RPMI 1640, and 1 M HEPES solution were purchased from Mediatech.

Isolation of human blood products

Neutrophils, serum, and plasma were isolated from the venous blood of healthy, medication-free, paid volunteers who gave informed consent. The Institutional Review Board of Stony Brook University approved this procedure. These procedures have been described in detail previously (15).

In vitro culture of U937 cells

U937 cells were originally obtained from the American Type Culture Collection and transfected with either the human C5a receptor or the empty plasmid vector as detailed previously (16). U937 cells were cultured at 37°C, 5% CO₂ in RPMI 1640 containing 10% FBS (HyClone) and 400 µg/ml active G418 (Invitrogen Life Technologies) and maintained at a density between 2×10^5 and 1.5×10^6 /ml.

Preparation of C-activated serum and plasma

Serum and citrated plasma (1 ml each) were incubated for 1 h at 37°C with 10 mg of zymosan. Particulate matter was removed by centrifugation ($15,000 \times g$) for 5 min at 4°C using a microfuge. Samples were then aliquoted and frozen at -20°C.

Chemotaxis assay

Cell movement was quantitated using a 48-well microchemotaxis chamber (NeuroProbe) and 5.0-µm pore size cellulose nitrate filters (purchased from NeuroProbe) as previously described (13). Cell movement was quantitated microscopically by measuring the distance in micrometers that the leading front of cells had migrated into the filter according to the method described by Zigmond and Hirsch (17). In each experiment, five fields per duplicate filter were measured at $\times 400$ magnification. The value of the background controls for random cell movement (cells responding to buffer) has been subtracted in all cases so that the data are presented as net movement in micrometers.

DBP binding assay

DBP was labeled with AlexaFluor 488 Protein Labeling kit (Molecular Probes) according to the manufacturer's instructions. U937-C5aR cells (50×10^6 cells/ml) in HBSS were pretreated with 100 nM ionomycin for 15 min at 37°C. The cells were then treated with protease inhibitor mixture (2 mM PMSF, 2 mM 1,10-phenanthroline, 0.5 mM E-64, and 0.5 mM Pefabloc), to prevent shedding of the DBP binding site, and incubated with 1 µM AlexaFluor 488-labeled DBP in HBSS containing 0.1% BSA (assay buffer) at 37°C for 45 min. After the incubation period, cells were washed in HBSS and resuspended in assay buffer and the relative fluorescence was measured using a Spectramax M2 (Molecular Devices). All samples were assayed in triplicate.

Coimmunoprecipitation, SDS-PAGE, and autoradiography

Purified DBP (200 µg) was radiolabeled using Iodobeads (Pierce) and Na¹²⁵I as previously described in detail (10). Neutrophils (100×10^6 cells) in 2 ml of HBSS were incubated with 0.4 µM ¹²⁵I-labeled DBP at 37°C for 60 min and then washed twice in HBSS. Cells were resuspended in lysis buffer (1% Triton X-100, 50 mM HEPES, 0.01% SDS) containing a protease inhibitor mixture (see *DBP binding assay*) and 1 mg/ml DNase I. After incubation at 37°C for 1 h, lysates were precleared with an irrelevant goat IgG for 1 h at 37°C, followed by 25 µl of protein G-Sepharose also for 1 h at 37°C. The lysates were then incubated with affinity-purified polyclonal goat anti-DBP or goat anti-CD44 for 1 h at 37°C. The immune complexes were isolated with 25 µl of protein G-Sepharose for 1 h at 37°C. Sepharose beads were washed twice in lysis buffer, and immunoreactive proteins were eluted from the protein G with SDS-PAGE sample buffer at 100°C for 10 min. Immunoprecipitates were separated by SDS-PAGE using an 8–16% gradient polyacrylamide gel (BioRad). The gel was fixed in 40% methanol and 10% acetic acid, rehydrated in dH₂O with 5% glycerol, dried, and exposed to x-ray film at -80°C.

Confocal microscopy

U937-Ca5R cells or neutrophils were suspended in HBSS plus 0.1% BSA at 5×10^6 /ml. Cells were stimulated with 100 nM ionomycin for 15 min at 37°C followed by treatment with the protease inhibitor mixture (to prevent shedding of the DBP binding site). Purified DBP (1 µM) was then added to the cells for 30 min at 37°C and then washed. Cells were incubated with mouse monoclonal anti-DBP (MAK-89), an irrelevant goat IgG, goat anti-CD44, or goat anti-annexin A2 for 30 min on ice. After washing, cells were incubated first with AlexaFluor 647-labeled donkey anti-goat IgG for 30 min on ice in the dark. Finally, after washing, AlexaFluor 488-labeled goat anti-mouse IgG was added for 30 min on ice in the dark. Cells were then washed twice in HBSS and fixed in 2% paraformaldehyde for 20 min then analyzed by confocal microscopy at $\times 400$ magnification. Cell pellets were resuspended in 20 µl of mounting medium/antifade reagent (Molecular Probes), mounted on glass slides with no. 1^{1/2} coverslips, and allowed to dry overnight. All samples were visualized using a Leica TCS SP2 confocal microscope, and images were processed using Adobe Photoshop.

Data analysis and statistics

A minimum of three experiments was performed for each assay. Results of several experiments were analyzed for significant differences among group means using ANOVA followed by Newman-Keul's multiple comparisons posttest using the statistical software program InStat (GraphPad).

Results

Cell surface molecules that may mediate the C5a chemotactic cofactor function of DBP were investigated using both U937-C5aR cells and neutrophils. In addition, both C-activated serum and C-activated plasma were used as chemoattractants (a source of C5a^{desArg} plus DBP) because U937-C5aR cells show significantly increased chemotaxis to C-activated serum as compared with C-activated plasma (15). This differential response, most prominently observed in U937-C5aR cells, is due to the presence of platelet-derived thrombospondin-1 in serum that facilitates the cochemotactic activity of DBP (15). Previous work from this lab using purified neutrophil plasma membrane preparations has demonstrated that DBP binds to a CSPG (10). To verify that a CSPG mediates the C5a chemotactic cofactor effect of DBP in U937-C5aR cells, chondroitin sulfate side chains were removed using either the enzyme chondroitinase AC (Fig. 1A) or cells were grown in 20 mM sodium chlorate (Fig. 1B), which inhibits sulfation of the glycosaminoglycan side chain. Fig. 1 demonstrates that either treatment significantly reduces U937-C5aR chemotaxis to an optimal concentration (2.5%) of C-activated serum but not to 2.5% C-activated plasma, indicating that a CSPG is required for DBP to function as a chemotactic cofactor. In addition, chemotaxis to 1 nM purified C5a (an optimal concentration), a DBP-independent process, was not altered by either chondroitinase (control, 57 ± 4.0 ; chondroitinase, 58 ± 2.6 µm/120 min) or chlorate (control, 62 ± 4.7 ; chlorate, 56 ± 4.8 µm/120 min) treatment of cells, indicating that treated cells possessed the capacity for a vigorous chemotactic response to C5a.

There are several different CSPGs on the surface of neutrophils and U937 cells, but CD44 is a particularly appealing candidate to be associated with the cochemotactic function of DBP. CD44 is a widely expressed type I transmembrane glycoprotein that has promiscuous binding properties and plays a role in migration of many cell types including leukocytes (18). To examine the function of CD44 in this process, cells were pretreated with an affinity-purified polyclonal anti-CD44 or an irrelevant goat IgG and then allowed to migrate toward either C-activated serum or C-activated plasma. Fig. 2 shows that anti-CD44 significantly reduces both U937-C5aR cell and neutrophil movement toward C-activated serum, but not C-activated plasma. Moreover, anti-CD44 treatment did not alter chemotaxis to 1 nM purified C5a for either U937-C5aR cells (control, 51 ± 3.9 ; anti-CD44, 46 ± 2.7 µm/120 min) or neutrophils

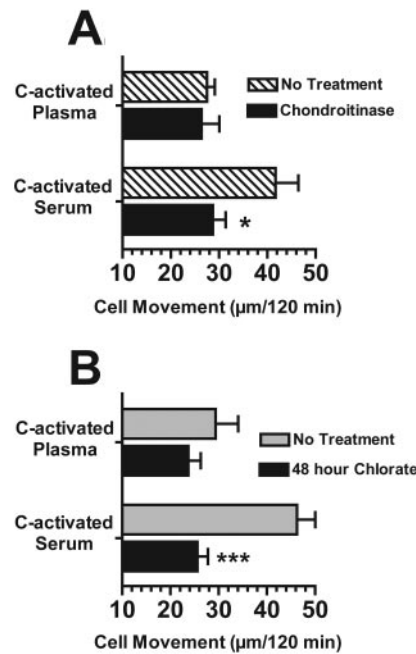


FIGURE 1. Removal of chondroitin sulfate side chains inhibits U937-C5aR cell movement to C-activated serum. *A*, Chondroitinase AC-treated cells; *B*, cells grown in sodium chlorate. U937-C5aR cells were treated with 2 mU/ml chondroitinase AC for 30 min at 37°C. Alternatively, U937-C5aR cells were grown in medium containing 20 mM sodium chlorate for 48 h. U937 cells (6×10^6 /ml) in chemotaxis buffer were allowed to respond to 2.5% C-activated serum or 2.5% C-activated plasma for 120 min at 37°C. Numbers represent mean \pm SEM, $n = 4$. Asterisks denote that cell movement was significantly less (*, $p < 0.05$ or ***, $p < 0.001$) than to the untreated control.

(control, 44 ± 3.9 ; anti-CD44, $44 \pm 5.3 \mu\text{m}/25 \text{ min}$), further indicating that the inhibitory effect is specific for the cochemotactic function of DBP. In addition, pretreatment of anti-CD44 with its corresponding antigenic peptide (i.e., Ag blocking peptide) completely reversed the effect of anti-CD44 on chemotaxis to C-activated serum (data not shown), confirming that the Ab mediates the inhibitory effect by binding to a CD44 peptide epitope. To determine whether DBP physically associates with CD44, coimmunoprecipitation studies were performed. For this experiment, ^{125}I -labeled DBP was incubated with neutrophils to allow the protein to bind surface ligands. After detergent solubilization and a preclearing step with goat IgG, cell lysates were immunoprecipitated with anti-CD44, anti-DBP (positive control), or goat IgG (negative control, data not shown). Fig. 3 demonstrates that radiolabeled DBP immunoprecipitates with anti-CD44 in neutrophils indicating a physical association between the two molecules. Furthermore, analysis of CD44 and cell-associated DBP by confocal microscopy shows a large overlap in the merged signal on the cell surface (Fig. 4). These results clearly demonstrate that DBP associates with cell surface CD44 and this glycoprotein plays a role in mediating the cochemotactic activity of DBP.

CD44 has been shown to associate with several cell surface macromolecules, some of which may participate in the formation of a DBP binding site/signaling complex. Annexin A2 (previously referred to as annexin II) is of particular interest because it has been shown to complex with CD44 in lipid rafts (19, 20), a unique cell surface microenvironment for receptor clustering and subsequent signaling. Annexin A2 is a widely expressed member of a family of Ca^{2+} -dependent phospholipid binding proteins and has been shown to participate in the formation of signaling complexes

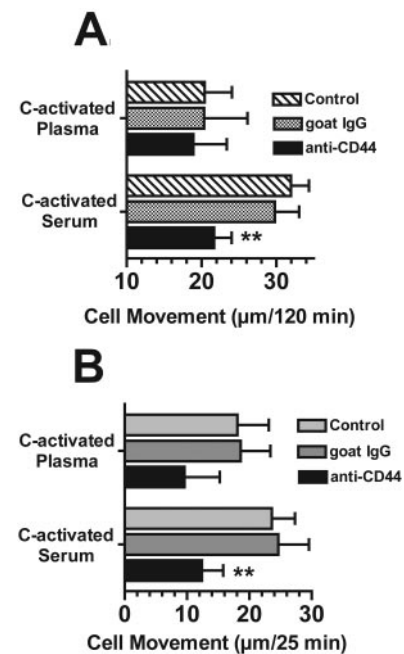


FIGURE 2. Anti-CD44 treatment inhibits neutrophil and U937-C5aR cell movement to C-activated serum. *A*, U937-C5aR cells; *B*, neutrophils. Neutrophils (4×10^6 /ml) or U937-C5aR cells (6×10^6 /ml) in chemotaxis buffer were treated for 15 min at 22°C with 20 $\mu\text{g}/\text{ml}$ anti-CD44 or an irrelevant goat IgG. Cells were then allowed to respond to either 2.5% C-activated serum or 2.5% C-activated plasma for either 25 (neutrophils) or 120 min (U937 cells) at 37°C. Numbers represent mean \pm SEM, $n = 5$. Asterisks denote that cell movement was significantly less ($p < 0.01$) than to the untreated control.

(21). To investigate the role of annexin A2 in mediating the cochemotactic activity of DBP, cells were pretreated with an affinity-purified polyclonal anti-A2 or an irrelevant goat IgG and then allowed to migrate toward either C-activated serum or C-activated plasma. Fig. 5 shows that anti-A2 significantly reduces U937-C5aR (Fig. 5A) and neutrophil (Fig. 5B) cell movement toward C-activated serum, but not C-activated plasma. Moreover, anti-A2 treatment did not alter chemotaxis to 1 nM purified C5a for either U937-C5aR cells (control, 48 ± 5.5 ; anti-A2, $46 \pm 4.0 \mu\text{m}/120 \text{ min}$) or neutrophils (control, 48 ± 3.6 ; anti-CD44, $48 \pm 4.4 \mu\text{m}/25 \text{ min}$). In addition, pretreatment of anti-A2 with its corresponding antigenic peptide (i.e., Ag blocking peptide) completely reversed the effect of anti-A2 on chemotaxis to C-activated serum (data not shown). Fig. 6 shows the analysis of annexin A2 and cell-associated DBP by confocal microscopy. The panel showing the merged signal (Fig. 6D) indicates considerable colocalization of the proteins on the cell surface. However, several attempts to immunoprecipitate DBP using anti-A2 failed, perhaps indicating that DBP-annexin A2 is a relatively weak, low avidity interaction (data not shown).

Finally, to determine whether anti-CD44 or anti-A2 blocks DBP binding to cells, U937-C5aR cells were pretreated with anti-CD44, anti-A2, both Abs, or an irrelevant goat IgG, and the binding of Alexafluor 488-labeled DBP was measured. Fig. 7A clearly demonstrates that either anti-CD44 or anti-A2 blocks DBP binding by almost 50%, whereas the combination of Abs reduces binding by $\sim 75\%$, a significantly greater reduction ($p < 0.01$) than either Ab alone. The added effect of dual Ab treatment on U937-C5aR cells was confirmed using the chemotaxis assay (Fig. 7B). A combination of anti-A2 and anti-CD44 showed an additional significant reduction ($p < 0.05$) in cell movement to C-activated serum over

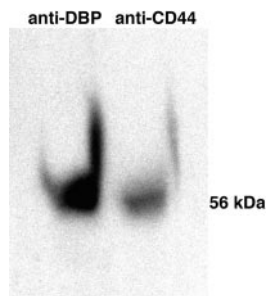


FIGURE 3. Anti-CD44 coimmunoprecipitates radiolabeled DBP from detergent-solubilized neutrophils. Neutrophils (100×10^6 cells) in 2 ml of HBSS were incubated with $0.4 \mu\text{M}$ ^{125}I -labeled DBP at 37°C for 60 min and then washed twice in HBSS. Cells were resuspended in lysis buffer (1% Triton X-100, 50 mM HEPES, 0.01% SDS) containing a protease inhibitor mixture and 1 mg/ml DNase I for 1 h at 37°C . The solubilized lysates were precleared with an irrelevant goat IgG for 1 h at 37°C , followed by $25 \mu\text{l}$ of protein G-Sepharose also for 1 h at 37°C . The lysates were then incubated with affinity-purified polyclonal goat anti-DBP or anti-CD44 for 1 h at 37°C , then immune complexes were isolated with $25 \mu\text{l}$ of protein G-Sepharose (1 h at 37°C). Samples were separated by SDS-PAGE using an 8–16% gradient polyacrylamide gel. The dried gel was exposed to x-ray film at -80°C .

anti-CD44 or anti-A2 alone, but had no effect on chemotaxis to C-activated plasma (Fig. 7B). These results indicate that both CD44 and annexin A2 are part of a cell surface DBP binding site complex and mediate the C5a chemotactic cofactor function of DBP.

Discussion

In this paper, several lines of evidence have been provided to demonstrate that CD44 and annexin A2 are part of the putative cell surface DBP binding site complex and function to mediate the chemotactic cofactor effect. The existence of a binding site complex has been inferred by functional (chemotaxis), structural (coimmunoprecipitation), and kinetic cell binding studies (10, 13–15). Presently, DBP has been shown to bind with low affinity to “scavenger-type” receptors such as CSPGs (10), low-density lipoprotein receptor family members (11, 12), and possibly CD36 (22). However, it is not known whether those receptors participate in mediating the cochemotactic signal; moreover, the exact composition and functional mechanism of this putative complex remain to be elucidated. The results presented in this study reveal that DBP binds to annexin A2 and CD44 and may use their signal transducing capacity to enhance chemotaxis to C5a/C5a^{desArg}. Annexin A2 and CD44 both have been shown to be associated with different aspects of cell movement. Annexin A2 can induce Ca^{2+} and phospholipid-dependent clustering of adapter proteins in lipid rafts that permit assembly of actin filaments (21). The cell surface CSPG CD44 has been shown to be the primary cellular receptor for hyaluronic acid and facilitates migration through the extracellular matrix (18). Moreover, CD44 associates with annexin A2 in lipid rafts on the extracellular face of the plasma membrane (19, 20), a unique membrane microenvironment for assembly of signaling complexes (23–25). Annexin A2 also has been reported to associate with a cell surface form of the vitamin D receptor in lipid rafts (26, 27), and it is presumed that DBP would interact with the plasma membrane vitamin D receptor to deliver its bound vitamin D sterol. These previous studies have provided the rationale to investigate whether CD44 and annexin A2 are potential members of the DBP binding site complex.

DBP clearly colocalizes with both CD44 (Figs. 3 and 4) and annexin A2 (Fig. 6) on the cell surface, but it is not known whether

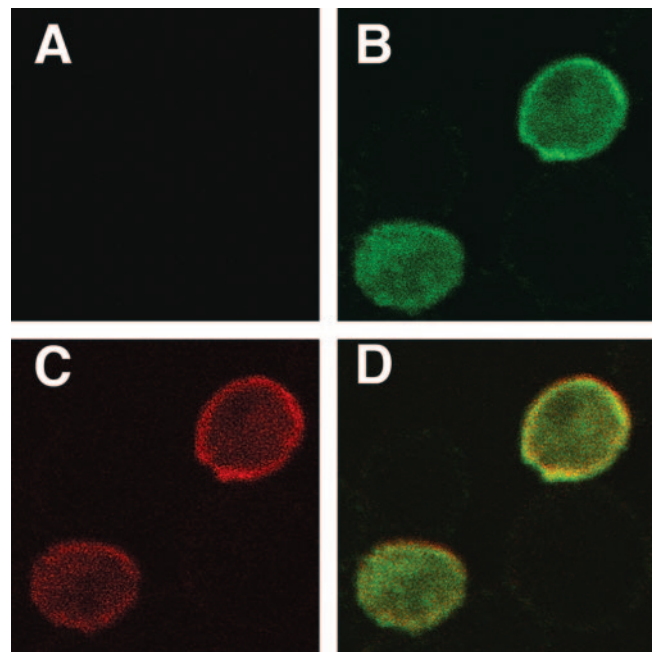


FIGURE 4. CD44 and DBP colocalize on the cell surface. U937-C5aR cells were suspended in HBSS plus 0.1% BSA at $5 \times 10^6/\text{ml}$. Cells were stimulated with 100 nM ionomycin for 15 min at 37°C and protease inhibitor mixture was added. Purified DBP ($1 \mu\text{M}$) was added to the cells for 30 min at 37°C and then washed. Cells were incubated with mouse monoclonal anti-DBP, goat anti-CD44, or an irrelevant goat IgG for 30 min on ice. After washing, cells were incubated with AlexaFluor 647-labeled donkey anti-goat IgG for 30 min on ice in the dark. Finally, after washing again, AlexaFluor 488-labeled goat anti-mouse IgG was added for 30 min on ice in the dark. Cells were washed twice in HBSS and fixed in 2% paraformaldehyde for 20 min then analyzed by confocal microscopy at $\times 400$ magnification. A, Goat IgG; B, DBP; C, CD44; D, merged image.

this occurs in lipid rafts. Furthermore, anti-CD44 and anti-A2 block DBP binding to cells (Fig. 7A) indicating that DBP binds both of these molecules. However, DBP coimmunoprecipitates with anti-CD44 (Fig. 3) but not with anti-A2, suggesting that the avidity of DBP binding to annexin A2 may be less than its association with CD44. A relatively weak interaction may not survive the somewhat harsh conditions of detergent solubilization used for coimmunoprecipitation. Nevertheless, Ab-blocking experiments (Figs. 2, 5, and 7B) demonstrate that both CD44 and annexin A2 appear to mediate the cochemotactic activity of DBP. Neutrophils and U937-C5aR cells treated with either anti-A2 or anti-CD44 show a significant reduction in chemotaxis to C-activated serum (DBP-dependent) but not to purified C5a (DBP-independent). In addition, pretreatment of either Ab with its corresponding antigenic peptide completely reversed the ability of anti-CD44 or anti-A2 to inhibit chemotaxis to C-activated serum (data not shown). The Abs had no effect on U937-C5aR migration to C-activated plasma, whereas anti-CD44 and anti-A2 inhibited neutrophil chemotaxis to C-activated plasma but the inhibition did not reach statistical significance. This unusual differential response to C-activated serum and plasma is due to the presence of platelet-derived thrombospondin-1 (present in serum but not in plasma) that facilitates the cochemotactic activity of DBP (15). Neutrophils have been shown to express thrombospondin-1 on their cell surface (28, 29) and thus migrate to C-activated plasma. However, the neutrophil response to C-activated plasma varies with each blood donor, whereas chemotaxis to C-activated serum produces very consistent results (G. Trujillo, L. A. McVoy, and R. R. Kew, unpublished

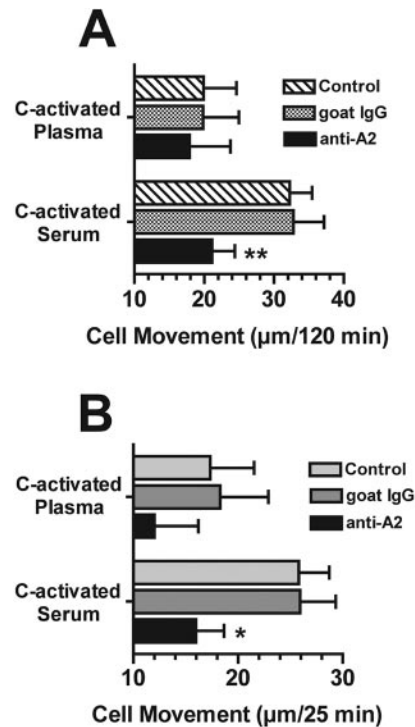


FIGURE 5. Anti-Annexin A2 treatment inhibits neutrophil and U937-C5aR cell movement to C-activated serum. *A*, U937-C5aR cells; *B*, neutrophils. Neutrophils ($4 \times 10^6/\text{ml}$) or U937-C5aR cells ($6 \times 10^6/\text{ml}$) in chemotaxis buffer were treated for 15 min at 22°C with 20 µg/ml anti-A2 or an irrelevant goat IgG. Cells were allowed to respond to either 2.5% C-activated serum or 2.5% C-activated plasma for either 25 (neutrophils) or 120 min (U937 cells) at 37°C. Numbers represent mean \pm SEM, $n = 5-6$. Cell movement was significantly less (*, $p < 0.05$ or **, $p < 0.01$) than to the untreated control.

observations). We believe that thrombospondin-1 bridges cell surface receptors and facilitates (i.e., accelerates) formation of the DBP binding site complex. However, currently there is no evidence that thrombospondin-1 directly interacts with either CD44 or annexin A2.

The abundant neutrophil serine protease elastase (30) is another molecule that has been associated with regulating the cochemotactic activity of DBP. Previously, we have shown that elastase releases the DBP binding site into the extracellular milieu, and highly specific elastase inhibitors prevent this shedding and also eliminate the C5a chemotactic cofactor effect of DBP (14). It is interesting to note that CD44 is shed by the action of a cell surface serine protease (31, 32), but the precise identity of the enzyme remains to be determined. Recently, we have discovered that specific inhibitors to the cell surface enzyme dipeptidyl peptidase I (also known as cathepsin C) eliminate U937-C5aR and neutrophil chemotaxis to C-activated serum but not to purified C5a (G. Trujillo and R. R. Kew, unpublished observations). This correlates with *in vivo* observations in dipeptidyl peptidase I knockout mice where neutrophils from these animals have a profound defect in chemotaxis to C-activated serum but migrate normally to CXCL8 (IL-8) (33). Thus, a picture is emerging to describe the mechanism by which DBP enhances chemotaxis to C5a/C5a^{desArg}. We believe that the formation of a DBP binding site complex is a highly regulated, sequential, multistep process involving several cell surface receptors and enzymes. This may explain why it takes at least 15–20 min after DBP is added to neutrophils before cochemotactic activity is detected (13, 34), a process that can be accelerated by

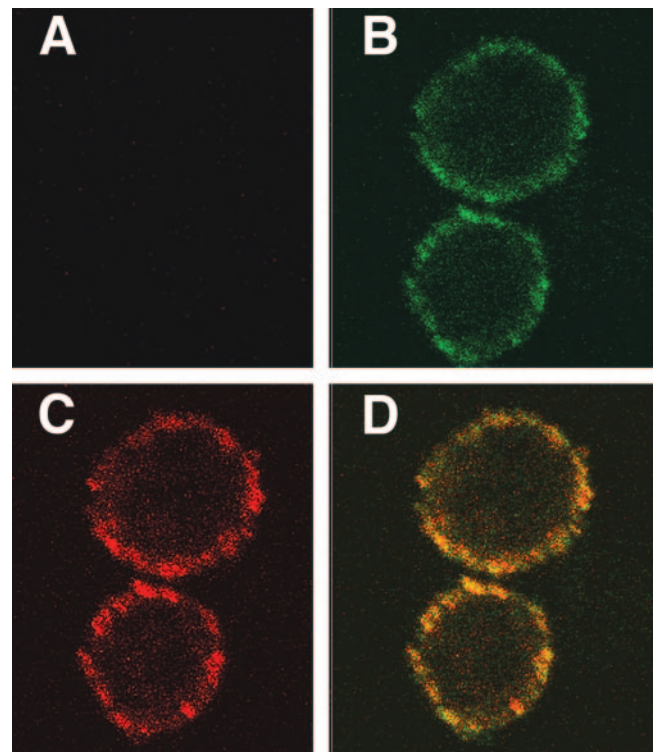


FIGURE 6. Annexin A2 and DBP colocalize on the cell surface. U937-C5aR cells were suspended in HBSS plus 0.1% BSA at $5 \times 10^6/\text{ml}$. Cells were stimulated with 100 nM ionomycin for 15 min at 37°C, and protease inhibitor mixture was added. Purified DBP (1 µM) was added to the cells for 30 min at 37°C and then washed. Cells were incubated with mouse monoclonal anti-DBP, goat anti-A2, or an irrelevant goat IgG for 30 min on ice. After washing, cells were incubated with AlexaFluor 647-labeled donkey anti-goat IgG for 30 min on ice in the dark. Finally, after washing again, AlexaFluor 488-labeled goat anti-mouse IgG was added for 30 min on ice in the dark. Cells were then washed twice in HBSS and fixed in 2% paraformaldehyde for 20 min then analyzed by confocal microscopy at $\times 400$ magnification. *A*, Control goat IgG; *B*, DBP; *C*, Annexin A2; *D*, merged image.

the addition of purified thrombospondin-1 (G. Trujillo, J. Zhang, and R. R. Kew, manuscript in preparation).

The C5a chemotactic cofactor function of DBP may be a specific example of a more generalized cellular response. We speculate that several soluble extracellular proteins (for example, complement C1q, fibrinogen, fibronectin, thrombospondin-1, DBP, vitronectin) may function as cell surface adapter molecules to bridge proteins that ordinarily would not interact with one another. In this way, they catalyze the assembly of novel signaling complexes, perhaps in lipid rafts, composed of several distinct proteins that serve as subunits of a nascent multifaceted receptor. Signaling would be initiated by the clustering of the adapter molecule. Although these soluble proteins do not function as classic high affinity ligands per se, they do act as *de facto* ligands required for assembly and subsequent signaling of the complex. Different adapter molecules could initiate assembly of unique receptor complexes and, in this way, could “mix and match” cell surface molecules to achieve a desired signal and cellular response. These complexes probably function transiently and could be terminated rapidly by posttranslational modifications such as proteolysis (cleavage and inactivation of a key protein and/or extracellular shedding of the complex), phosphorylation, dephosphorylation, deglycosylation, etc. Furthermore, one could speculate that disease conditions could adversely affect the normal functioning of these

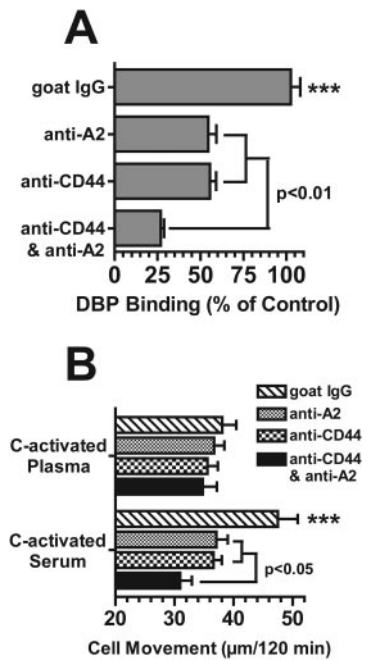


FIGURE 7. Anti-CD44 and anti-annexin A2 inhibit DBP binding to cells. **A**, Binding. U937-C5aR cells (50×10^6 cells/ml) cells in HBSS were pretreated with 100 nM ionomycin for 15 min at 37°C. Cells were treated for 15 min at 22°C with 20 µg/ml anti-A2, anti-CD44, either Ab, or an irrelevant goat IgG then washed. Cells were then treated with a protease inhibitor mixture and incubated with 1 µM AlexaFluor 488-labeled DBP in HBSS containing 0.1% BSA (assay buffer) at 37°C for 45 min. After the incubation period, cells were washed in HBSS and resuspended in assay buffer, and the relative fluorescence was measured. Numbers represent the mean \pm SEM ($n = 5$) of AlexaFluor 488-labeled DBP binding to cells expressed as a percentage of control (binding to untreated cells). Asterisks denote that binding was significantly greater ($p < 0.001$) than all other samples. The dual Ab-treated cells bound significantly less ($p < 0.01$) DBP than either anti-A2 or anti-CD44 treatment alone. **B**, Chemotaxis. U937-C5aR cells (6×10^6 /ml) in chemotaxis buffer were treated for 15 min at 22°C with 20 µg/ml anti-A2, anti-CD44, either Ab, or an irrelevant goat IgG. Cells were then allowed to respond to either 2.5% C-activated serum or 2.5% C-activated plasma for 120 min at 37°C. Numbers represent mean \pm SEM, $n = 5-6$. Asterisk denotes that cell movement was significantly greater than all other samples. The dual Ab-treated cells migrated significantly less ($p < 0.05$) than either anti-A2 or anti-CD44 treatment alone.

complexes. Excessive or improper production of inflammatory mediators could trigger inadvertent generation or premature termination of physiological complexes or, conceivably, formation of aberrant pathological signaling complexes. Nevertheless, given our current level of understanding, any proposed mechanism to explain the C5a chemotactic cofactor effect of DBP is tentative.

It has been suggested for many years that C5a is a paramount chemoattractant in vivo (35, 36). More recent in vitro evidence has further strengthened this concept, demonstrating that C5a functions as a primary “end-target” chemoattractant (37, 38). Therefore, cofactors that significantly enhance the chemotactic activity of C5a, such as DBP, also would be very important physiologically. DBP is a ubiquitous protein in vivo, it has been detected in almost all body fluids at levels capable of inducing cochemotaxis to C5a (39). Likewise, CD44 and annexin A2 are widely expressed on the extracellular face of the plasma membrane of many cell types including most leukocytes (18, 21). Thus, DBP would be present any time C5a is produced and would be available to mediate its cochemotactic effect via interaction with CD44 and an-

nexin A2. There may be other yet to be identified molecules in the DBP binding site complex that are required for cochemotactic activity. However, the model system used in this study allows for precise identification of factors that mediate this function of DBP, because U937-C5aR cells will respond only to C5a/C5a^{desArg} (16), and the chemotactic response of these cells to C-activated serum is largely dependent on DBP (15). Therefore, the U937-C5aR cell model allows for in vitro screening of candidate molecules (using blocking Abs or specific inhibitors) that subsequently can be verified using peripheral blood neutrophils. This experimental approach should enable the complete identification of the DBP binding site complex and then facilitate elucidation of the signaling mechanisms triggered when DBP binds to the complex. This knowledge will clarify the multiple functions of DBP in cell physiology.

Disclosures

The authors have no financial conflict of interest.

References

- Kew, R. R., and R. O. Webster. 1988. Gc-globulin (vitamin D binding protein) enhances the neutrophil chemotactic activity of C5a and C5a des Arg. *J. Clin. Invest.* 82: 364–369.
- Perez, H. D., E. Kelly, D. Chenoweth, and F. Elfman. 1988. Identification of the C5a des Arg cochemotaxin: homology with vitamin D-binding protein (group-specific component globulin). *J. Clin. Invest.* 82: 360–363.
- Petrini, M., A. Azzara, G. Carulli, F. Ambrogi, and R. M. Galbraith. 1991. 1,25-Dihydroxycholecalciferol inhibits the chemotactic activity of Gc (vitamin D binding protein). *J. Endocrinol. Invest.* 14: 405–408.
- Metcalfe, J. P., A. B. Thompson, G. L. Gossman, K. J. Nelson, S. Koyama, S. I. Rennard, and R. A. Robbins. 1991. Gc-globulin functions as a cochemotaxin in the lower respiratory tract: a potential mechanism for lung neutrophil recruitment in cigarette smokers. *Am. Rev. Respir. Dis.* 143: 844–849.
- Binder, R., A. Kress, G. Kan, K. Herrmann, and M. Kirschfink. 1999. Neutrophil priming by cytokines and vitamin D binding protein (Gc-globulin): impact on C5a-mediated chemotaxis, degranulation and respiratory burst. *Mol. Immunol.* 36: 885–892.
- Zwahlen, R. D., and D. R. Roth. 1990. Chemotactic competence of neutrophils from neonatal calves: functional comparison with neutrophils from adult cattle. *Inflammation* 14: 109–123.
- Senior, R. M., G. L. Griffin, H. D. Perez, and R. O. Webster. 1988. Human C5a and C5a des Arg exhibit chemotactic activity for fibroblasts. *J. Immunol.* 141: 3570–3574.
- Piquette, C. A., R. Robinson-Hill, and R. O. Webster. 1994. Human monocyte chemotaxis to complement-derived chemotaxins is enhanced by Gc-globulin. *J. Leukocyte Biol.* 55: 349–354.
- White, P., and N. Cooke. 2000. The multifunctional properties and characteristics of vitamin D-binding protein. *Trends Endocrinol. Metab.* 11: 320–327.
- DiMartino, S. J., and R. R. Kew. 1999. Initial characterization of the vitamin D binding protein (Gc-globulin) binding site on the neutrophil plasma membrane. Evidence for a chondroitin sulfate proteoglycan. *J. Immunol.* 163: 2135–2142.
- Nykjaer, A., D. Dragun, D. Walther, H. Vorum, C. Jacobsen, J. Herz, F. Melsen, E. I. Christensen, and T. E. Willnow. 1999. An endocytic pathway essential for renal uptake and activation of the steroid 25-(OH) vitamin D₃. *Cell* 96: 507–515.
- Nykjaer, A., J. C. Fyfe, R. Kozyraki, J. R. Leheste, C. Jacobsen, M. S. Nielsen, P. J. Verroust, M. Aminoff, A. de la Chapelle, S. K. Moestrup, et al. 2001. Cubilin dysfunction causes abnormal metabolism of the steroid hormone 25(OH) vitamin D₃. *Proc. Natl. Acad. Sci. USA* 98: 13895–13900.
- Kew, R. R., J. A. Fisher, and R. O. Webster. 1995. Co-chemotactic effect of Gc-globulin (vitamin D binding protein) for C5a: transient conversion into an active co-chemotaxin by neutrophils. *J. Immunol.* 155: 5369–5374.
- DiMartino, S. J., A. B. Shah, G. Trujillo, and R. R. Kew. 2001. Elastase controls the binding of the vitamin D-binding protein (Gc-globulin) to neutrophils: a potential role in the regulation of C5a co-chemotactic activity. *J. Immunol.* 166: 2688–2694.
- Trujillo, G., and R. R. Kew. 2004. Platelet-derived thrombospondin-1 is necessary for the vitamin D binding protein (Gc-globulin) to function as a chemotactic cofactor for C5a. *J. Immunol.* 173: 4130–4136.
- Kew, R. R., T. Peng, S. J. Dimartino, D. Madhavan, S. J. Weinman, D. Cheng, and E. R. Prossnitz. 1997. Undifferentiated U937 cells transfected with chemoattractant receptors: a model system to investigate chemotactic mechanisms and receptor structure/function relationships. *J. Leukocyte Biol.* 61: 329–337.
- Zigmond, S., and J. Hirsch. 1973. Leukocyte locomotion and chemotaxis: new methods for evaluation and demonstration of a cell-derived chemotactic factor. *J. Exp. Med.* 137: 387–410.
- Pure, E., and C. A. Cuff. 2001. A crucial role for CD44 in inflammation. *Trends Mol. Med.* 7: 213–221.
- Oliferenko, S., K. Paiha, T. Harder, V. Gerke, C. Schwarzler, H. Schwarz, H. Beug, U. Gunther, and L. A. Huber. 1999. Analysis of CD44-containing lipid

- rafts: Recruitment of annexin II and stabilization by the actin cytoskeleton. *J. Cell Biol.* 146: 843–854.
20. Merrifield, C. J., U. Rescher, W. Almers, J. Proust, V. Gerke, A. S. Sechi, and S. E. Moss. 2001. Annexin 2 has an essential role in actin-based macropinocytotic rocketing. *Curr. Biol.* 11: 1136–1141.
 21. Rescher, U., and V. Gerke. 2004. Annexins: unique membrane binding proteins with diverse functions. *J. Cell Sci.* 117: 2631–2639.
 22. Kanda, S., Y. Mochizuki, Y. Miyata, H. Kanetake, and N. Yamamoto. 2002. Effects of vitamin D₃-binding protein-derived macrophage activating factor (Gc-MAF) on angiogenesis. *J. Natl. Cancer Inst.* 94: 1311–1319.
 23. Brown, D. A., and E. London. 1998. Functions of lipid rafts in biological membranes. *Annu. Rev. Cell. Dev. Biol.* 14: 111–136.
 24. Gomez-Mouton, C., R. A. Lacalle, E. Mira, S. Jimenez-Baranda, D. F. Barber, A. C. Carrera, A. C. Martinez, and S. Manes. 2004. Dynamic redistribution of raft domains as an organizing platform for signaling during cell chemotaxis. *J. Cell Biol.* 164: 759–768.
 25. Sitrin, R. G., D. R. Johnson, P. M. Pan, D. M. Harsh, J. Huang, H. R. Petty, and R. A. Blackwood. 2004. Lipid raft compartmentalization of urokinase receptor signaling in human neutrophils. *Am. J. Respir. Cell Mol. Biol.* 30: 233–241.
 26. Mizwicki, M. T., J. E. Bishop, C. J. Olivera, J. Huhtakangas, and A. W. Norman. 2004. Evidence that annexin II is not a putative membrane receptor for 1 α ,25(OH)₂-vitamin D₃. *J. Cell. Biochem.* 91: 852–863.
 27. Huhtakangas, J. A., C. J. Olivera, J. E. Bishop, L. P. Zanello, and A. W. Norman. 2004. The vitamin D receptor is present in caveolae-enriched plasma membranes and binds 1 α ,25(OH)₂-vitamin D₃ in vivo and in vitro. *Mol. Endocrinol.* 18: 2660–2671.
 28. Kries, C., M. La Fleur, C. Ménard, R. Paquin, and A. D. Beaulieu. 1989. Thrombospondin and fibronectin are synthesized by neutrophils in human inflammatory joint disease and in a rabbit model of in vivo neutrophil activation. *J. Immunol.* 143: 1961–1968.
 29. Suchard, S. J., L. A. Boxer, and V. M. Dixit. 1991. Activation of human neutrophils increases thrombospondin receptor expression. *J. Immunol.* 147: 651–659.
 30. Lee, W. L., and G. P. Downey. 2001. Leukocyte elastase: physiological functions and role in acute lung injury. *Am. J. Respir. Crit. Care Med.* 164: 896–904.
 31. Bazil, V., and J. L. Strominger. 1994. Metalloprotease and serine protease are involved in cleavage of CD43, CD44, and CD16 from stimulated human granulocytes: induction of cleavage of L-selectin via CD16. *J. Immunol.* 152: 1314–1322.
 32. Mollinedo, F., B. Martinmartin, C. Gajate, and P. A. Lazo. 1998. Physiological activation of human neutrophils down-regulates CD53 cell surface antigen. *J. Leukocyte Biol.* 63: 699–706.
 33. Adkison, A. M., S. Z. Raptis, D. G. Kelley, and C. T. Pham. 2002. Dipeptidyl peptidase I activates neutrophil-derived serine proteases and regulates the development of acute experimental arthritis. *J. Clin. Invest.* 109: 363–371.
 34. Kew, R. R., K. W. Mollison, and R. O. Webster. 1995. Binding of Gc globulin (vitamin D binding protein) to C5a or C5a des Arg is not necessary for chemotactic activity. *J. Leukocyte Biol.* 58: 55–58.
 35. Hugli, T. E. 1984. Structure and function of the anaphylatoxins. *Springer Semin. Immunopathol.* 7: 193–219.
 36. Czermak, B. J., A. B. Lentsch, N. M. Bless, H. Schmal, H. P. Friedl, and P. A. Ward. 1998. Role of complement in in vitro and in vivo lung inflammatory reactions. *J. Leukocyte Biol.* 64: 40–48.
 37. Foxman, E. F., E. J. Kunkel, and E. C. Butcher. 1999. Integrating conflicting chemotactic signals. Role of memory in leukocyte navigation. *J. Cell Biol.* 147: 577–588.
 38. Foxman, E. F., J. J. Campbell, and E. C. Butcher. 1997. Multistep navigation and the combinatorial control of leukocyte chemotaxis. *J. Cell Biol.* 139: 1349–1360.
 39. Cooke, N. E., and J. G. Haddad. 1989. Vitamin D binding protein (Gc-globulin). *Endocr. Rev.* 10: 294–307.