DEMONSTRATION OF A SPECIFIC RECEPTOR FOR HUMAN C5a ANAPHYLATOXIN ON MURINE MACROPHAGES*

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Human C5a anaphylatoxin is a 74 residue glycopolypeptide cleaved from the amino terminus of the fifth component of human complement (C5) during complement activation (1). The C5a fragment serves as a potent mediator of the acute inflammatory response. Its ability to induce mast cell histamine release, enhanced vascular permeability, and contraction of smooth muscle account for its spasmogenicity (2–4). More importantly, human C5a is the most potent complement-derived effector of human neutrophil function yet described. In vitro, C5a is capable of stimulating neutrophil chemotaxis, inducing exocytosis of lysosomal granular enzymes, enhancing formation of toxic oxygen species, and promoting enhanced neutrophil adherence and/or autoaggregation (5–9).

These neutrophil responses ensue when C5a binds to specific receptors on the neutrophil surface (10). These cells bear ~200,000 C5a receptors, which are characterized by an apparent $K_d$ of 1–3 nM. Recent investigations have demonstrated that neutrophil-bound radioiodinated C5a ($^{125}$I-C5a) is readily degraded to small peptides and/or amino acids and is eventually released from the cell. Still other studies suggest that proteolytic degradation of C5a occurs only after cellular internalization of the ligand (11, 12).

When C5a is formed in serum or plasma, it is rapidly and irreversibly converted to its des Arg-74 derivative (C5ades-Arg) by serum carboxypeptidase N (E.C. 3.4.17.3) (13). Although human C5ades-Arg is nearly devoid of spasmogenic activity, it retains the ability to stimulate neutrophil responses such as chemotaxis and degranulation (6, 7). Additionally, human C5ades-Arg is capable of binding to the neutrophil C5a receptor but with a 50- to 100-fold lower affinity than C5a (6, 10).

In addition to their neutrophil-related biological activities, C5a and/or C5ades-Arg
have also been demonstrated to promote a variety of monocyte and macrophage responses, including enhancement of migration or chemotaxis (14–16), induction of lysosomal enzyme secretion (17), and polarization (18). We assume that these cellular responses, like those of the neutrophil, result from binding of C5a to specific cellular receptors. To confirm this hypothesis, we have characterized the binding interactions of radiolabeled human C5a with both adherent cells isolated from the peritoneal cavity of C3H/HeJ mice and the murine macrophage cell line designated P388D1.

Materials and Methods

Preparation of Human C5a and C5a Derivatives. Human C5a anaphylatoxin was purified to homogeneity from zymosan-activated human serum containing 1 M ε-aminocaproic acid by the methods of Fernandez and Hugli (19). The naturally occurring derivative C5adec Arg was isolated from zymosan-activated human serum in the absence of the carboxypeptidase inhibitor (1). An additional C5a derivative, termed C5a-(1-69), which consists of the N-terminal 69 residues of C5a, was prepared by carboxypeptidase Y digestion of C5adec Arg as previously described (10).

Human C5a was radiolabeled with Na 125I (Amersham Corp., Arlington Heights, IL) by a solid phase lactoperoxidase-glucose oxidase method (Enzymobead; Bio-Rad Laboratories, Richmond, CA) using conditions recommended by the manufacturer (Bio-Rad Technical Bulletin 1060). Free 125I and damaged radiolabeled C5a were separated from antigenically reactive 125I-C5a by immunoaffinity chromatography on a 1.0-ml column of rabbit anti-C5a IgG-Sepharose 4B. Native 125I-C5a was eluted with 0.2 M glycine-HCl, pH 2.3, and immediately neutralized with 1 M K2HPO4. The specific activity of such preparations was typically 20–40 μCl/μg. Radiolabeled C5a was judged to have retained its native character by the fact that it was >90% bound by an excess of rabbit anti-human C5a immunoglobulin in solid phase radioimmunoassays (20) and >85% bound by human neutrophils.

Mice. Male mice (C3H/St), 8–12 wk of age, were obtained from the mouse breeding colony at Scripps Clinic and Research Foundation. Mice were maintained on Wayne Lab-Blox F6 pellets (Allied Mills, Inc., Chicago, IL) and chlorinated water acidified with HCl to a pH of 3.0 (21).

Cell Isolation and Culture. Resident peritoneal cells were obtained by peritoneal lavage with 3.0 ml of a sterile balanced salt solution (BSS) supplemented with 100 U of penicillin G/ml, 100 μg of streptomycin/ml, and 0.5 U (USP) of sodium heparin/ml. Thioglycollate-induced peritoneal cells were similarly obtained 6 d after intraperitoneal injection of thioglycollate. Before use, all peritoneal cells were irradiated with 1,000 rad from a Gamma cell 40 small animal irradiator (Atomic Energy of Canada Ltd., Ottawa, Canada). This irradiator uses a cesium 137 source emitting a central dose-rate of 100 rad/min. Differential cell counts were performed on each cell preparation before performing 125I-C5a binding assays. Typically, 10–30% of resident peritoneal cells were esterase positive, whereas 90–95% of the thioglycollate-induced cells were esterase positive. The number of peritoneal cells used was adjusted for percent cells of monocytoid histology. Cells of this morphological type were radiation-resistant, esterase-positive, adherent antigen-presenting cells. For these reasons they will be referred to as "macrophages."

Murine spleen cell suspensions were prepared as previously described (22). These cells were depleted of adherent cells by passage over columns of Sephadex G-10 (23). The lymphocyte population recovered from this procedure typically contained <0.12% esterase-positive cells.

In selected experiments, murine resident peritoneal cells were depleted of Ia-positive cells by treatment with monoclonal anti-IaK antibodies and complement. Monoclonal anti-IaK was obtained as a culture supernatant from hybridoma 10-2.16 developed in the laboratory of Dr. L. A. Herzenberg at Stanford University. This antibody is of the γ2b subclass. It reacts with Ia specificities of the r, f, and s haplotype in addition to IaK (24). Treatment with this supernatant followed by addition of guinea pig complement (Colorado Serum Co., Denver, CO) typically killed ~16% of resident peritoneal cells from C3H/St mice.

The cell lines P388 and P388D1 were kindly provided by the Salk Institute for Biological Studies, La Jolla, CA. P388 cells were cultured in Dulbecco's minimum essential medium (high
glucose) supplemented with 50 µM 2-mercaptoethanol, 20 mM Hepes, and 10% heat-inactivated fetal calf serum (FCS). P388D1 cells were cultured in RPMI 1640 supplemented with 5% heat-inactivated FCS. Additionally, both types of media were supplemented with glutamine, nonessential amino acids, and antibiotics as described for lymphocyte culture (22). Human peripheral blood neutrophils were prepared from the heparin-anticoagulated venous blood of healthy male volunteers according to standard methods (25).

125I-C5a Cell Binding Assays. Cellular binding of 125I-C5a was assessed by previously published methods (10). Briefly, 125I-C5a was diluted to an appropriate concentration (0.3-60 nM) in Hanks’ balanced salt solution containing 0.5% gelatin (HBSS-G) (Microbiological Associates, Walkersville, MD) and incubated with test cells suspended in HBSS-G. Incubation volumes were routinely 100 µl, and binding reactions were carried out in 1.5-ml conical polypropylene microfuge tubes (Cole Scientific, Calabasas, CA). To terminate the binding reaction, cells were rapidly pelleted by centrifugation at 11,000 g in a Beckman Microfuge B (Beckman Instruments, Inc., Palo Alto, CA) for 30 s. To determine the amount of cell-bound 125I-C5a, one-half the supernatant solution plus the material remaining in the microfuge tube (residual supernatant and cell pellet) was counted as a paired sample in a Packard Autogamma Spectrometer (Packard Instrument Co., Downers Grove, IL) with 70% efficiency. The amount of cell-bound 125I-C5a was then calculated from the formula given previously (10). Nonspecific binding of 125I-C5a to polypropylene tubes was <1% when assays were performed in this manner.

Identification of 125I-C5a Degradation Products. Molecular sieve chromatography was utilized to identify 125I-C5a degradation products released by murine macrophages upon prolonged incubation at 37°C. For these experiments, peritoneal cells from C3H/HeJ mice (1 × 107/ml in HBSS-G) were incubated with 1 nM 125I-C5a for 20 min at 37°C to permit ligand binding. After removal of unbound 125I-C5a by washing, the cells were resuspended in HBSS-G and incubated for an additional 2 h at 37°C. Cells were then pelleted by centrifugation, and 100 µl of the incubation supernatant was removed and chromatographed on a 0.7 × 30-cm column of Bio Gel P-100 (100-200 mesh; Bio-Rad Laboratories). The column was eluted at a flow rate of 4.65 ml/h with phosphate-buffered saline, pH 7.4, and 0.3-ml fractions were collected. Radioactive content of individual fractions was assessed by counting gamma emissions as above.

Results

Time Course and Temperature Dependence of 125I-C5a Binding. The time course of 125I-labeled human C5a binding to resident murine peritoneal macrophages at both 4°C and 37°C is shown in Fig. 1. Cellular uptake of the ligand was equally rapid at both temperatures. At 4°C, binding was maximal after 45 min, and steady-state binding was demonstrable with incubation for up to 120 min. When binding was carried out at 37°C, maximal cellular uptake of 125I-C5a was observed after 20 min incubation. With more prolonged incubation at this temperature, the amount of cell-bound radioisotope steadily decreased, being barely detectable after 120 min of incubation. This type of kinetic profile, i.e., ligand uptake followed by cellular release of radioisotope, has been consistently observed when 125I-C5a is incubated with neutrophils at 37°C (11, 12) or when target cells bind and subsequently degrade bioactive polypeptides (reviewed in 26).

To determine if murine macrophages degrade cell-associated 125I-C5a, cells that had been incubated with 125I-C5a for 20 min at 37°C were washed and incubated in the absence of C5a for an additional 2 h at 37°C. When supernatants from these cultures were subjected to gel filtration chromatography on Bio Gel P-100 (Fig. 2) only ~15% of the released radioisotope eluted as intact 125I-C5a. The vast majority of the radioactive material eluted between 11 and 13.5 ml, a position whose elution volume was consistent with that of mono-iodotyrosine or small peptides. Further analysis of this low molecular weight fraction demonstrated that it did not bind to
Fig. 1. Time course of binding of 125I-labeled human C5a to C3H/HeJ resident peritoneal macrophages. Cells were suspended at a density of 5 × 10⁵ macrophages (esterase-positive cells)/ml in HBSS-G and incubated a final concentration of 0.3 nM 125I-C5a at 4°C (O) or 37°C (©). Duplicate 100-µl aliquots were removed at the indicated times, and the amount of cell-associated radiolabel determined as described in Materials and Methods. Results of duplicate determinations in a single experiment are shown. Three additional experiments gave comparable results.

Fig. 2. Bio Gel P-100 column chromatography of radiolabel released from murine (C3H/HeJ) resident peritoneal macrophages. Cells (1 × 10⁷/ml in HBSS-G) were incubated with 1 nM 125I-C5a for 20 min at 37°C, washed to remove unbound ligand, resuspended in HBSS-G at a density of 1 × 10⁷/ml, and incubated for an additional 2 h at 37°C. Cells were pelleted by centrifugation, and a 100-µl aliquot of the incubation supernatant was chromatographed on a 0.7 × 30-cm column of P-100 resin. Two separate experiments gave identical results.

either human neutrophils or to freshly-prepared murine peritoneal macrophages. Additionally, it was not bound by an excess of rabbit anti-human C5a immunoglobulin.

Concentration Dependence of 125I-labeled Human C5a Binding to Murine Macrophages. Incubation of murine resident peritoneal macrophages with varying concentrations of 125I-C5a for 20 min at 37°C demonstrated a saturable binding interaction, with a nonspecific binding component at elevated ligand concentrations (Fig. 3). A Scatchard plot of these data (Fig. 4) suggested the existence of a single class of high affinity cellular receptors as well as low affinity (nonspecific) binding interactions. Linear regression analysis (correlation coefficient = 0.998) of the high affinity binding data
MACROPHAGE C5a RECEPTOR

Fig. 3. Concentration dependence of 125I-labeled human C5a binding to C3H/HeJ resident peritoneal macrophages. Cells were suspended at a density of $1 \times 10^7$ macrophages/ml in HBSS-G and incubated with varying concentrations of 125I-C5a for 20 min at 37°C. Cell-bound 125I-C5a was determined in triplicate as described. Each data point represents the mean value of triplicate determinations in three separate experiments. Nonspecific binding has not been subtracted from the uptake data presented.

Fig. 4. Scatchard plot of data presented in Fig. 3, showing binding of 125I-labeled human C5a to murine peritoneal macrophages.

provided an estimate of the apparent $K_d$ of the murine peritoneal macrophage C5a receptor as 3 nM. The average number of C5a receptors per cell was estimated to be $2 \times 10^5$.

Ligand Specificity of the Macrophage C5a Receptor. Incubation of murine peritoneal macrophages with a fixed amount of 125I-C5a and increasing amounts of unlabeled C5a resulted in progressive diminution of 125I-C5a binding (Fig. 5). The ligand specificity of the murine macrophage C5a receptor was further confirmed by the observation that the unlabeled C5a derivatives C5a$_{des\text{ Arg}}$ and C5a-(1-69) also proved capable of inhibiting cellular binding of 125I-C5a. These two ligands, which display lower affinity binding than C5a, produced half maximal inhibition of C5a binding at approximate concentrations of $1.6 \times 10^{-6}$ M and $2.4 \times 10^{-5}$ M, respectively. Qualitatively similar results have been obtained with the human neutrophil C5a receptor (6, 27). The chemotactic oligopeptide N-formyl-methionyl-leucyl-phenylalanine (N-f-MLF), which also binds specifically to macrophages (28, 29), did not
CHENOWETH, GOODMAN, AND WEIGLE

Figure 5. Ligand specificity of $^{125}$I-labeled human C5a binding to C3H/HeJ resident peritoneal macrophages. Macrophages ($2.5 \times 10^5$/ml in HBSS-G) were simultaneously incubated for 20 min at 37°C with radiolabeled C5a (1 nM) and varied amounts of unlabeled C5a (○), C5aa replacement (■), C5a-(1-69) (▲), or N-F-MLF (□). The amount of $^{125}$I-C5a bound to cells in the absence or presence of competing ligand was determined as described. Each data point represents the mean value of duplicate determinations in two experiments.

Figure 6. Cellular specificity of $^{125}$I-labeled human C5a binding to various types of murine cells. Resident peritoneal macrophages (○), thioglycollate-elicited peritoneal macrophages (■), or splenic lymphocytes prepared by passage through Sephadex G-10 (□) were incubated with varying concentrations of $^{125}$I-C5a for 20 min at 37°C. Each data point represents the mean value obtained from triplicate determinations in a single experiment. Two additional experiments gave comparable results. Nonspecific binding, determined by computer-assisted graphical analysis (31), was subtracted from each curve.

displace $^{125}$I-C5a from murine macrophages even though it was present in a $10^5$ molar excess, thus indicating that C5a receptors and oligopeptide receptors exist as separate entities on the macrophage surface. Similar findings have been obtained with neutrophils (30).

Cellular Specificity of the C5a Receptor. The cellular specificity of $^{125}$I-labeled human C5a binding was analyzed by determining the concentration-dependent binding of $^{125}$I-C5a to a variety of cells. As shown in Fig. 6, saturable binding of C5a was demonstrable with resident peritoneal macrophages as well as cells elicited by the prior administration of thioglycollate. Both types of cells exhibited half-maximal
MAcrophage C5a Receptor

Table I

| Cell type                              | Origin          | Apparent $K_d^*$ | Receptor number$\dagger$ |
|----------------------------------------|-----------------|------------------|--------------------------|
| Resident peritoneal macrophage         | Murine (C3H/HeJ) | 2-3 nM           | 200,000                  |
| Thioglycollate-induced peritoneal macrophage | Murine (C3H/HeJ) | 2-3 nM           | 40,000-50,000           |
| Splenic lymphocytes                    | Murine (C3H/HeJ) | 0§               |                          |
| P388D1                                 | Murine          | 2-3 nM           | 80,000-100,000          |
| P388                                   | Murine          | 0§               |                          |

$* K_d$ estimated from half-maximal uptake of $^{125}$I-C5a.

$\dagger$ Receptor number estimated from saturable portions of $^{125}$I-C5a uptake curves.

§ No detectable specific binding of $^{125}$I-C5a.

uptake of $^{125}$I-C5a at a concentration of 20 ng/ml (2.5 nM), implying that the C5a receptors of each cell type exhibited the same apparent $K_d$. On the other hand, resident macrophages displayed $\sim 2 \times 10^5$ receptors/cell, whereas the thioglycollate-induced macrophages possessed only $4-5 \times 10^4$ C5a receptors per cell. Splenic lymphocytes, rendered free of adherent cells by passage through Sephadex G-10, did not bind $^{125}$I-C5a. Thus, the C5a receptor is expressed by macrophages, but is not found in significant numbers on murine splenic lymphocytes.

Additional studies of this type have also been conducted to determine if C5a receptors exist on murine macrophage cell lines. The results of these investigations are summarized in Table I. Our findings indicate that the cell line P388D1, but not its parent P388, expressed C5a receptors. The apparent $K_d$ of the C5a receptor expressed by P388D1 was 2 nM, and 8-10 $\times 10^4$ C5a binding sites were detectable on each cell.

We have also assessed the binding of $^{125}$I-C5a to unseparated and Ia-depleted murine cells. To accomplish this, Ia-positive cells were removed from the normal peritoneal macrophage population by lysis with specific anti-Ia antiserum and complement. Evaluation of the binding of 1 nM $^{125}$I-C5a for 20 min at 37°C by normal and Ia-depleted cells demonstrated that the normal mixed cell population bound 36.3 ± 1.8 (mean ± SD) fmol of $^{125}$I-C5a, whereas the macrophage population depleted of Ia-positive cells bound 47.6 ± 1.2 (mean ± SD) fmol of $^{125}$I-C5a. This difference was judged to be statistically significant ($P < 0.001$).

Discussion

Our current investigations clearly demonstrate that murine macrophages, like human peripheral blood neutrophils (10), possess a specific receptor for the complement-derived C5a anaphylatoxin. The characteristics of the murine macrophage C5a receptor are quantitatively similar to those of the human neutrophil (10), i.e., the apparent $K_d$ is estimated to be 2-3 nM, and each cell bears $\sim 200,000$ C5a receptors. Radiolabeled human C5a binds rapidly to macrophages obtained from the peritoneal cavity of C3H mice. The rate of association of the ligand with these cells appears to be equally rapid whether binding takes place at 4°C or 37°C, implying receptor-mediated uptake of the biologically active factor. When incubations are conducted entirely at 4°C, steady-state binding is obtained. In contrast, when binding interactions take place at 37°C, uptake and time-dependent release of radiolabel is observed, suggesting that a temperature-dependent process(es), such as internalization, is re-
sponsible for these phenomena (12). Therefore, murine peritoneal macrophages appear to behave like human neutrophils, being capable of degrading cell-bound $^{125}$I-C5a.

Although we have not specifically attempted to prove that murine macrophages internalize $^{125}$I-C5a like human neutrophils, we have clearly demonstrated that the macrophage is capable of degrading C5a to its constituent amino acids and/or small peptides. Supernatants recovered from cells incubated with $^{125}$I-C5a for 2 h at 37°C were chromatographically resolved on a molecular sieve resin and shown to consist predominantly of low molecular weight radiolabeled fragments. The fact that these low molecular weight species were not bound by either freshly-prepared human neutrophils, murine macrophages, or anti-C5a immunoglobulin indicated that they were neither biologically nor antigenically reactive. Thus, macrophages, like neutrophils, are able to degrade and inactivate cell-bound C5a. Based on our own observations with neutrophils and the study of polypeptide hormones conducted by many other investigators (26), it is likely that macrophage internalization of $^{125}$I-C5a precedes the degradative-inactivation process(es).

Resident macrophages isolated from the peritoneal cavity of C3H mice demonstrate a single class of high affinity C5a receptors. Scatchard analysis of $^{125}$I-C5a concentration-dependent binding data indicates that these receptors display an average $K_d$ of 3 nM, and that there are $\sim 2 \times 10^5$ receptors on each cell. These values correlate closely with those observed for human neutrophils (10). Some low affinity or nonspecific binding interactions are also demonstrated by this type of analysis. The fact that a 100-fold molar excess of unlabeled C5a completely blocks binding of $^{125}$I-C5a suggests that these low affinity interactions may represent a second population of low affinity receptors rather than simply nonspecific binding.

The C5a receptors of murine macrophages, like their neutrophil counterparts, exhibit a high degree of ligand specificity (6, 10). Unlabeled human C5a and its derivatives C5a$_{des}$ Arg$_2$ and C5a-(1-69) are all capable of inhibiting $^{125}$I-C5a binding to the macrophage receptor. The relative affinities of each unlabeled ligand, i.e., C5a$>$C5a$_{des}$ Arg$_2$$>$C5a-(1-69), are comparable to those demonstrated with human neutrophils (6, 10). These findings imply that the macrophage C5a receptor may be structurally analogous to the human neutrophil C5a receptor. If this is true, then the macrophage C5a receptor may consist of both a ligand binding domain that interacts with the N-terminal region of C5a and an activation binding domain that specifically interacts with the C-terminal portion of C5a (6).

The macrophage C5a receptor is found not only on murine resident peritoneal macrophages, but is also demonstrable on thioglycollate-induced peritoneal cells and cultured cell lines. In all instances, the apparent $K_d$ of the receptor is nearly identical, being 1-3 nM. Adherent cells from different sources appear to differ predominantly with regard to the number of receptors expressed. For example, resident peritoneal macrophages have four to five times as many receptors as their thioglycollate-induced counterparts and approximately twice as many receptors as the P388D$_1$ cell line. Interestingly, the cell line P388, which is the parent of P388D$_1$, fails to express a detectable number of C5a receptors. The fact that C5a receptor density may vary widely, whereas the apparent $K_d$ remains constant, is substantiated by the observation that human alveolar macrophages, guinea pig alveolar macrophages, and human peripheral blood monocytes display C5a receptors whose apparent $K_d$ values are 1-3 nM. However, each of these cell types express differing numbers of receptors, i.e.,
MACROPHAGE C5a RECEPTOR

Human alveolar macrophages, \(3 \times 10^5\); guinea pig alveolar macrophages, \(0.5 \times 10^5\); and human monocytes, \(1 \times 10^5\) (unpublished observations).

We have observed that lysis of Ia-positive cells with specific anti-Ia antisera and complement actually increases subsequent binding of \(^{125}\)I-C5a to the viable peritoneal cells remaining in culture. The magnitude of the enhancement of \(^{125}\)I-C5a specific uptake is consistent with the fact that such lytic procedures removed the 10–20% of Ia-positive cells, which would be normally expected in the resident peritoneal macrophage population, and implies that Ia-negative cells are largely responsible for the observed binding of C5a. This conclusion is substantiated by the finding that the Ia-negative cell line P388D1 also binds \(^{125}\)I-C5a.

Although other investigators have demonstrated that C5a or a similar polypeptide in activated serum is capable of promoting macrophage cellular responses (14–18), we have chosen to focus on a new biological activity ascribable to C5a. As we have demonstrated, C5a promotes macrophage-dependent enhancement of the immune response. The close correspondence between the observed dose-response profile for this phenomenon and that of \(^{125}\)I-C5a binding to macrophages implies that the binding of C5a to its macrophage receptor eventuates in a significant biological response and further confirms the specific nature of the macrophage C5a receptor.

Summary

Human C5a anaphylatoxin is known to be a potent mediator of the acute inflammatory response. It serves to trigger a wide variety of neutrophil responses after binding to a specific cellular receptor. We have now demonstrated that this bioactive glycopolypeptide is also bound to a specific receptor found on murine resident peritoneal macrophages, thioglycollate-induced exudate macrophages, and the murine cell line P388D1. The apparent \(K_d\) of the C5a receptor for each cell type is \(2 \text{ nM}\), but each cell expresses a differing number of C5a receptors. Resident macrophages appear to have an average of \(2 \times 10^5\) binding sites per cell, whereas thioglycollate-induced cells have only \(4–5 \times 10^4\) binding sites. The continuous cell line P388D1 is intermediate between these two cell types, exhibiting \(8–10 \times 10^4\) C5a receptors per cell. Neither murine lymphocytes nor the parent cell line P388 displays a measurable number of C5a receptors. Macrophage receptor-C5a binding interactions are followed by cellular uptake and degradation of \(^{125}\)I-C5a, much as is observed with neutrophils.

As demonstrated in another paper, binding of C5a to macrophages results in augmentation of the primary humoral immune response as well as enhancement of mixed lymphocyte reactions. These observations suggest that C5a should not only be considered as an acute inflammatory mediator but as an immunopotentiating modulator as well, thus serving as a critical link between complement activation and subsequent immune responses.

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