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Complement Component C5 Recruits Neutrophils in the Absence of C3 during Respiratory Infection with Modified Vaccinia Virus Ankara

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Efficient leukocyte migration is important for an effective host response to viral infection and the development of adaptive immunity. The poxvirus strain modified vaccinia virus Ankara (MVA), a safe and efficient viral vector, rapidly induces chemokine expression and respiratory recruitment of leukocytes, which is unique among vaccinia viruses. In addition to chemokines, the complement system contributes to the attraction and activation of different types of leukocytes. Using a murine model of intranasal infection, we show in this study that MVA-induced neutrophil recruitment depends on complement component C5. Remarkably, we find that C5 mediates neutrophil recruitment to the lung, even in the absence of the central complement component C3. Our findings argue for complement C5 activation during MVA infection of the lung via a C3-independent pathway, which enables rapid recruitment of neutrophils. The Journal of Immunology, 2015, 194: 000–000.

The complement system has long been considered a key component of the innate immune response with a crucial role in the defense against invading pathogens. Comprising of >30 serum proteins and surface receptors, the complement system is an intricately connected proteolytic cascade that rapidly generates local inflammatory and cytolytic responses. Activation occurs via three main pathways—classical, lectin, and alternative (1)—all of which converge on the activation of the central C3 component, opsonizing the target with C3b and forming C5 convertase, which activates the terminal C5 component by proteolytic cleavage. Activation of C5 releases the potent inflammatory mediator C5a and generates C5b, initiating the formation of the lytic membrane attack complex.

Complement is present at high concentrations in serum, and synthesis of complement components primarily takes place in the liver. Complement components are also secreted locally in tissues (2) and produced by cells of the immune system including dendritic cells (3) and macrophages (4, 5). The prevalence of complement in local tissues, coupled with its independence from host gene expression, allows the complement system to rapidly induce local immune responses through the generation of inflammatory mediators. Activation of complement may be an important mechanism for the rapid induction of innate immune responses that lead to the recruitment of leukocytes. Autocrine activation of C5 by alveolar macrophages is required for the optimal generation of CXC and CC chemokines (6); similarly, activation of the alternative pathway on the surface of neutrophils creates an amplification loop, perpetuating further neutrophil recruitment (7). Additionally, as C5a is directly chemotactic for leukocytes, it may form part of a complex milieu of spatially and temporally overlapping signals that coordinate leukocyte homing (8).

Viral infection potentially activates both the classical and alternative pathways of the complement system. As a direct consequence, deposition of complement on the surface of virions enhances uptake by phagocytosis and can interfere with receptor interactions, virus entry, and uncoating (9). The second consequence of complement activation is the release of anaphylotoxins C3a and C5a, both of which induce local proinflammatory responses such as the recruitment of leukocytes and the release of lysosomal enzymes and vasoactive amines.

Modified vaccinia virus Ankara (MVA) is a highly attenuated, replication-deficient strain of vaccinia virus (VACV) derived by serial passage in chicken embryo fibroblast cells (10). MVA has lost many of the host modulatory factors that are encoded in wild-type orthopoxviruses (11), including the virally encoded complement control protein that is a known virulence factor (12, 13). Previously, we have shown that MVA, but not other VACV strains, induces robust innate immune responses including the early recruitment of leukocytes into the lung (14). During the early stages of MVA infection, neutrophils make up a large portion of infiltrating leukocytes, which are recruited by a mechanism involving CCR1 (15). In this study, we show that MVA infection leads to complement component C5-dependent recruitment of neutrophils to the lung, which is independent of the central complement component C3.

Materials and Methods

Mice

C57BL/6NCrl mice (B6), B10.D2-Hc1 H2d H2-T18'/osSnJ mice, and the complement component C5-deficient B10.D2-Hc1 H2d H2-T18'/osSnJ mice were purchased from Charles River Laboratories. C3-deficient mice (C3 -/-)
backcrossed for 10 generations to the C57BL/6 background and wild-type control mice were obtained from Marcela Pecka as recently described (16). Sera were routinely checked for the presence of the complement C3 component by Western blot (Supplemental Fig. 1). All mice were maintained under specific pathogen-free conditions. Mice were housed in a temperature- and light-controlled room (21–23°C, 55 ± 3% relative humidity, 12-h light/12-h dark cycle) and were fed a standard rodent diet with sterilized water ad libitum. All animal procedures in this study were in accordance with German Animal Welfare law and the institutional Animal Welfare Committee. All experiments were approved by the Government of Upper Bavaria (Munich, Germany).

Infection of mice, bronchoalveolar lavage, and C5 neutralization

Mice were anesthetized and inoculated intranasally with 10⁷ PFU MVA or equivalent volumes of endotoxin-free PBS (Biochrom). At the indicated time points, mice were euthanized and cells recovered from the lungs by washing four times with 1 ml very low endotoxin RPMI 1640 (Biochrom) containing 10% PBS and 10 mM EDTA. The first wash was stored separately, and after removal of cells by centrifugation, levels of C5a were analyzed by ELISA, and the presence of C5 was checked by Western blot (Supplemental Fig. 2). Cells from all four washes were combined for flow cytometric analysis. For in vivo C5 neutralization studies, mice were injected i.p. with 500 μg anti-mouse C5 mAb (clone 295108; R&D Systems (Bio-Rad). C5 was performed with polyclonal goat anti-mouse C3 Ab (MP Biotechnology) and anti-rat IgG-HRP (Santa Cruz Biotechnology). Recombinant mouse C3 was performed with monoclonal rat anti-mouse C5a Ab (clone MOPC-21; BioXCell) 24 h prior to infection.

Flow cytometric analysis

Cells collected in the bronchoalveolar lavage (BAL) fluid were washed in PBS and then stained with Aqua Zombie (BioLegend) for exclusion of dead cells. After incubation, cells were washed with PBS containing 1% FBS, and nonspecific binding was blocked by incubation with anti-CD16/32 Abs before incubation with fluorescently tagged Abs: CD11b-Brilliant Violet 421 (clone M1/70) and Ly6G-allophycocyanin (clone 1A8; BioLegend). After staining, samples were run on a MACSQuant Y Vy flow cytometer (Miltenyi Biotec). Data were analyzed using FlowJo software (Tree Star). Neutrophils were gated as CD11bhigh, Ly6G+ cells according to Blomgran and Ernst (17), and CD11c was used to verify discrimination between alveolar macrophages (CD11bhigh/CD11c−) (Supplemental Fig. 3).

ELISA

Levels of C5a in the BAL fluid were quantified using the Mouse Complement Component C5a DuoSet ELISA Kit (R&D Systems); the assay was carried out according to the manufacturers’ instructions.

Western blotting

BAL fluid was mixed with Laemmli sample buffer and run on a 4–20% Criterion TGX Stain Free Gel (Bio-Rad), allowing rapid fluorescent detection of total proteins before and after protein transfer on the basis of the incorporation of tris(hydroxymethyl)aminomethane (Tris)-buffered saline-SDS sample buffer. After incubation, cells were washed with PBS containing 1% FBS, and nonspecific binding was blocked by incubation with anti-CD16/32 Abs before incubation with fluorescently tagged Abs: CD11b-Brilliant Violet 421 (clone M1/70) and Ly6G-allophycocyanin (clone 1A8; BioLegend). After staining, samples were run on a MACSQuant Vy flow cytometer (Miltenyi Biotec). Data were analyzed using FlowJo software (Tree Star). Neutrophils were gated as CD11bhigh, Ly6G+ cells according to Blomgran and Ernst (17), and CD11c was used to verify discrimination between alveolar macrophages (CD11bhigh/CD11c−) (Supplemental Fig. 3).

Statistical analysis

GraphPad Prism 5 (GraphPad Software) was used for statistical analysis. Statistical significance was calculated by ANOVA using Bonferroni post hoc test; a p value <0.05 was considered significant.

Results

Neutrophil recruitment during MVA infection is mediated by complement component C5

VACV activates both the classical (13) and alternative (18) complement pathways, and complement is known to play an important role in poxvirus immunity (19). Activation of the terminal C5 component leads to the liberation of the small peptide fragment C5a, which is a potent immune activator and chemoattractant, particularly for neutrophils (20). To examine the role of complement component C5 in MVA-triggered recruitment of neutrophils, C57BL/6 mice were treated with an Ab that neutralizes C5 and intranasally infected with MVA 24 h later. Neutrophil recruitment to the lungs of anti-C5 treated mice was reduced 9-fold compared with the untreated control mice at 12 h postinfection (p.i.) (Fig. 1A, 1B).

This corresponded with a 2.7-fold increase in the concentration of C5a in the BAL fluid of MVA-infected mice compared with mock infected mice, which was significantly reduced through application of the neutralizing anti-C5 mAb (Fig. 1C).

Additionally, we assessed neutrophil recruitment in the B10 mouse strain in comparison with the congenic C5-deficient strain. MVA infection of B10 mice induced neutrophil recruitment to the lung, which was more pronounced than levels typically observed in C57BL/6 mice. C5-deficient mice consistently presented with lower levels of infiltrating neutrophils up to 12 h p.i., confirming a role for C5 in the early recruitment of neutrophils during respiratory infection of mice with MVA (Fig. 2A, 2B). As before, the concentration of C5a in the BAL fluid was increased in MVA-infected mice, and as expected, no C5a could be detected in BAL fluids from C5-deficient mice (Fig. 2C).

MVA-induced respiratory migration of leukocytes occurs independently of complement component C3

Both the classical and alternative complement activation pathways converge on the cleavage of the central C3 component, which is responsible for crucial effector functions including opsonization and activation of the terminal C5 component via formation of a C5 convertase.

FIGURE 1. MVA-induced neutrophil recruitment is impaired in anti-C5-treated mice. B6 mice were i.p. injected with a C5-neutralizing Ab, 24 h prior to intranasal infection with MVA or the equivalent volume of PBS. Cells collected by BAL, at 12 h p.i., were analyzed by flow cytometry. (A) Representative dot plots from flow cytometric analysis showing levels of infiltrating CD11b+Ly6G+ neutrophils (PMN). (B) Summary of the analysis. Columns represent mean cell number of each gated cell population ± SD (PBS, n = 3; MVA, n = 5). (C) C5a concentrations in BAL fluids were determined by ELISA 12 h p.i. Data are group means ± SD (PBS groups, n = 3; MVA groups, n ≥ 5). **p < 0.01, ***p < 0.001.
To access the role of these major pathways of complement activation in the immune response to MVA, we examined neutrophil recruitment to the lungs in C3-deficient mice. Surprisingly, we observed a significant increase in the number of infiltrating neutrophils in C3-deficient mice, which was also evident at 16 h p.i. (Fig. 3A, 3B). C5a concentrations in the lungs were significantly increased by MVA infection in B6 and C3-deficient mice to a similar level (Fig. 3C), potentially indicating that C5 is functioning independently of C3.

**C3 is dispensable for C5-mediated recruitment of neutrophils**

Previously, it has been reported that phagocytic cells can activate complement component C5 independently of C3 (21). Our results indicated that C5 plays a role in MVA-induced neutrophil recruitment and that C3 does not appear to be necessary for this process. Consequently, to test whether C5 drives MVA-induced neutrophil recruitment independently of the C3 component, C3-deficient mice were treated with an Ab that neutralizes C5 and intranasally infected with MVA. Flow cytometric analysis revealed that the number of neutrophils present in the lung were significantly reduced in anti-C5 mAb–treated mice compared with C3-deficient mice treated with the same amount of a matched isotype control Ab (Fig. 4A, 4B). Similar to wild-type C57BL/6 mice (Fig. 1C), the anti-C5 mAb significantly reduced C5a concentrations in the BAL fluid of MVA-infected C3-deficient mice to levels detected in mock-infected, C3-deficient mice (Fig. 4C).

**Discussion**

C3-independent activation of the complement C5 component has been known for some time (21–25). However, the relevance of these C3-independent pathways in vivo is not well studied and was
C5 RECRUITS NEUTROPHILS INDEPENDENTLY OF C3

FIGURE 4. Neutralization of C5 prevents MVA-induced neutrophil recruitment in C3-deficient mice. C57BL/6 C3-deficient mice were i.p. injected with a C5-neutralizing Ab 24 h prior to intranasal infection with MVA or the equivalent volume of PBS. Cells collected by BAL, at 12 h p.i., were analyzed by flow cytometry. (A) Representative dot plots from flow cytometric analysis showing levels of infiltrating CD11b^+Ly6G^+ neutrophils (PMN). (B) Summary of the analysis. Columns represent mean cell number of each gated cell population ± SD (PBS, n = 3; MVA, n = 6). (C) C5a concentrations in BAL fluids were determined by ELISA 12 h p.i. Data are group means ± SD (PBS groups n = 3, MVA groups n = 7). ***p < 0.001.

never considered in the context of viral infections. Activation of C5 independently of C3 has been observed during cerebral malaria infection, in which it makes a critical contribution to the development of disease (26). We discovered that C5 functions independently of C3 to induce early neutrophil recruitment during infection with MVA, a nonreplicating viral vector known to activate the innate immune system (27). C5 may be activated by proteolytic enzymes such as serine proteases released from phagocytic cells (21), as we observed no differences in C3-deficient mice treated with antithrombin III (data not shown), ruling out thrombin-mediated activation (25). Activation of C5 by locally released proteases could serve to rapidly amplify the local immune response, as C5a induces inflammatory cytokine expression and is chemotactic for leukocytes.

To explore this possibility, we investigated MVA-induced neutrophil recruitment in anti-C5–treated mice. Levels of infiltrating neutrophils were significantly reduced in treated mice, indicating that C5 activation products play a critical role in the recruitment of neutrophils during MVA infection. These findings were confirmed in C5-deficient B10 mice; however, the reduction was less pronounced. Notably, levels of neutrophil recruitment in the B10 strain were consistently higher than levels observed in C57BL/6 mice, which could indicate additional and/or more dominant mechanisms that may compensate somewhat for the lack of C5. In contrast, complement component C3 was not required for the early recruitment of neutrophils that appeared to be enhanced in C3-deficient mice. This potentially fits with reports that apoptotic cells opsonized with the C3 breakdown product iC3b exert anti-inflammatory effects on monocytes and dendritic cells (28). A second possibility is that in the absence of C3-opsonized targets, neutrophil effector functions such as phagocytosis and recirculation may be reduced. Additionally, in the absence of C3, formation of the membrane attack complex may be impaired, allowing the persistence of virus-infected cells, which could exacerbate the inflammatory response.

Although we revealed an important role for C5 in neutrophil recruitment up to 12 h p.i., it is likely that as the inflammatory response develops, other more persistent signals such as those provided by chemokines exert the dominant effects. To this extent, chemokine receptor CCR1 also plays an important role in MVA-induced neutrophil recruitment, and the effects of CCR1 deficiency persist for longer (15). In addition to being directly chemotactic, C5a acts synergistically to enhance CC and CXC chemokine production (29). Therefore, we speculate that C5 bridges the gap inducing rapid recruitment of neutrophils while simultaneously amplifying chemokine expression to enable prolonged recruitment and the maturation of the inflammatory immune response.

The complement system plays an important role in poxvirus immunity, and it is interesting to note the seemingly divergent roles of the complement components at different stages of the host immune response. We found that C3 does not play an important role in the early recruitment of leukocytes during the inflammatory response to MVA, possibly because C5 appears to be able to function independently of C3. However, C3 is important at later stages of the immune response, as it is required for Ab-mediated neutralization of poxviruses (30) and the development of T cell responses (31). This importance of the central complement activation pathways in the adaptive immune response was demonstrated by Moulton et al. (19), who showed that C3 is required to survive mousepox infection. In contrast, C5 appears to be dispensable for the development of the adaptive immune response. The primary immune response to cowpox infection was altered in C5-deficient mice, with exacerbated inflammation and increased pathology. However, though the inflammatory response was affected, the development of the adaptive immune response was not, as upon reinfection these differences were no longer apparent (32). Instead C5 appears to be rapidly activated independently of C3 exerting an influence over the development of the innate immune response by participating in local recruitment of neutrophils and may function independently of host Abs to induce clearance of VACV (33). Therapeutic treatment with a specially engineered C5a agonist, EP67, completely protects from lethal influenza infection (34); therefore, the rapid activation of the C5 component induced by MVA infection may go some way toward explaining the short-term protection provided by MVA. Consequently, in future studies, it would be interesting to test whether C5 plays a role in the rapid protection provided by MVA against a lethal challenge.

The results presented in this study confirm our previous findings that MVA induces early immigration of leukocytes to the lung and show that during the inflammatory response to MVA, C5 acts independently of C3 to drive early neutrophil recruitment.

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Disclosures
The authors have no financial conflicts of interest.

References


