Impaired mast cell-dependent natural immunity in complement C3-deficient mice

Andrey P. Prodeus*, Xiaoning Zhou†, Marcus Maurer*, Stephen J. Galli† & Michael C. Carroll*†

* Departments of Pathology, Harvard Medical School and † Beth Israel Deaconess Medical Center, Boston, Massachusetts, 02115, USA

The complement system is widely regarded as essential for normal inflammation, not least because of its ability to activate mast cells.1–3 However, recent studies have called into question the importance of complement in several examples of mast cell-dependent inflammatory responses.4–9 To investigate the role of complement in mast cell-dependent natural immunity, we examined the responses of complement-deficient mice10,11 to caecal ligation and puncture12,13, a model of acute septic peritonitis12,13 that is dependent on mast cells and tumour necrosis factor-α (TNF-α). We found that C4- or C3-deficient mice10,11 were much more sensitive to caecal ligation and puncture than wild-type (WT) controls (100% versus 20% in 24-h mortality, respectively). C3-deficient mice also exhibited reductions in peritoneal mast cell degranulation, production of TNF-α, neutrophil infiltration and clearance of bacteria. Treating the C3-deficient mice with purified C3 protein enhanced activation of peritoneal mast cells, TNF-α production, neutrophil recruitment, opsonophagocytosis of bacteria and resistance to caecal ligation and puncture, confirming that the defects were complement-dependent. These results provide formal evidence that complement activation is essential for

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*† Correspondence and requests for materials should be addressed to H.Y.Z. (e-mail: hzho@bchm.pcm. tmc.edu).

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The complement system is widely regarded as essential for normal inflammation, not least because of its ability to activate mast cells. However, recent studies have called into question the importance of complement in several examples of mast cell-dependent inflammatory responses. To investigate the role of complement in mast cell-dependent natural immunity, we examined the responses of complement-deficient mice to caecal ligation and puncture, a model of acute septic peritonitis that is dependent on mast cells and tumour necrosis factor-α (TNF-α). We found that C4- or C3-deficient mice were much more sensitive to caecal ligation and puncture than wild-type (WT) controls (100% versus 20% in 24-h mortality, respectively). C3-deficient mice also exhibited reductions in peritoneal mast cell degranulation, production of TNF-α, neutrophil infiltration and clearance of bacteria. Treating the C3-deficient mice with purified C3 protein enhanced activation of peritoneal mast cells, TNF-α production, neutrophil recruitment, opsonophagocytosis of bacteria and resistance to caecal ligation and puncture, confirming that the defects were complement-dependent. These results provide formal evidence that complement activation is essential for
the full expression of innate immunity in this mast cell-dependent model of bacterial infection.

Caecal ligation and puncture (CLP) produced substantially (P < 0.0001) more mortality in C4-/– mice19 or complement C3 (C3-/–) mice20 than in wild-type mice, particularly within the first 24 h following the procedure (100% versus 20% mortality, respectively) (Fig. 1). The enhanced susceptibility of the C3-/– mice to CLP was due to the absence of functional C3, as reconstitution of the C3-deficient animals with a single intraperitoneal injection of 0.5 mg purified human C3 protein (HuC3) reduced the 24-h mortality from 100 to 40% (Fig. 1). Previous studies had demonstrated that human C3 could be substituted for mouse C3 in vitro21 and in vivo22 and that this dose, which results in blood levels in C3-/– mice that are roughly one-quarter of that in the blood of wild-type mice, was rapidly absorbed into the circulation following intraperitoneal (i.p.) injection (our unpublished results). Protection by C3 protein replacement was temporary, however, as by 48 h after CLP mortality in the HuC3-treated C3-/– mice had increased to 60%. This result was not unexpected because exogenous C3 is rapidly catabolized in the C3-deficient mice (our unpublished results).

Peritoneal mast cells recovered from the C3-deficient mice 1 or 3 h after CLP exhibited evidence of degranulation, but significantly less than that observed in the wild-type mice (Fig. 2). Thus, by 1 h after CLP, more than 75% of peritoneal mast cells identified in cytospins from wild-type mice were moderately or extensively degranulated compared to fewer than 50% of the mast cells in the C3-/– mice (P < 0.001). By contrast peritoneal mast cells in HuC3-reconstituted C3-/– mice exhibited levels of degranulation after CLP that were similar to those of mast cells in the wild-type mice (Fig. 2).

C3-/– (n = 10) and wild-type (n = 9) mice had similar numbers of total peritoneal cells (4.0 ± 0.2 versus 4.0 ± 0.3 × 106 per mouse, P > 0.05) and total peritoneal mast cells (3.3 ± 0.5 versus 4.7 ± 0.7 × 106 per mouse, P > 0.05) at baseline before CLP. However, the number of peritoneal mast cells that can be recovered by peritoneal lavage after CLP is less than can be obtained from the unmanipulated peritoneal cavity. Moreover, mast cell cytokine production does not always correlate with degranulation21. We therefore sought to use another, more relevant index of mast cell activation in this model. One hallmark of mast cell activation is the rapid release of TNF-α16,17, the cytokine that has been implicated as an important mediator in mast cell-dependent models of natural immunity, including CLP18 and other examples of bacterial infection23. When we measured the levels of TNF-α in peritoneal lavage fluids that were collected after CLP and assayed by enzyme-linked immunosorbent assay (ELISA), we found significantly (P < 0.0001) lower TNF-α levels at both 1 h (158 ± 24 pg ml–1 (n = 13) versus 400 ± 45 pg ml–1 (n = 14) and 3 h (218 ± 35 pg ml–1 (n = 13) versus 663 ± 53 pg ml–1 (n = 14) in the C3-/– versus wild-type animals (Fig. 3a). Reconstitution with C3 protein restored TNF-α levels to those of wild-type mice, at both 1 h (344 ± 40 pg ml–1 (n = 5) and 3 h (521 ± 61 pg ml–1 (n = 5)) following CLP (Fig. 3a).

Neutrophils represent less than 1% of the total cells in the peritoneal cavity of untreated C3-/– or wild-type mice. In wild-type mice, neutrophils account for 50% of peritoneal cells at 1 h and 90% of cells at 3 h following CLP (Fig. 3b). In contrast, significantly (P < 0.0001) fewer neutrophils were found in the peritoneal cavity of C3-/– mice at the same time points after CLP (1% and 45%, respectively). TNF-α importantly contributes to mast cell-dependent neutrophil recruitment, as pretreatment of mice with an antibody specific to TNF-α can substantially reduce mast cell-dependent infiltration of neutrophils, both in the skin at sites of IgE and antigen challenge19 and in the peritoneal cavity in models of bacterial infection18 or immune complex deposition20,21. Enhancement of neutrophil infiltration by TNF-α in these settings may reflect a number of different actions of this cytokine, such as upregulation of E selection on venular endothelial cells, induction of neutrophil chemo-attractants such as interleukin-8 (IL-8), and the direct upregulation and activation of integrins on leukocytes19,20.

As assessed in cytocentrifuge preparations, the delayed and reduced infiltration of neutrophils into the peritoneal cavity of C3-/– mice after CLP was associated with a less efficient clearance of bacteria than in wild-type mice, by either 1 or 3 h (Fig. 4a–e). Because cytocentrifuge preparations of each of the CLP-treated C3-/– mice revealed reduced numbers of neutrophils and large numbers of bacteria (Fig. 4b–e), whereas
few or no bacteria were seen in the preparations from the wild-type or HuC3-reconstituted C3−/− mice (Fig. 4a, c, d, f), the peritoneal lavage fluids (n = 5) from each group were pooled and cultured overnight. About 20-fold more colony-forming units (CFU) of Escherichia coli were identified in the peritoneal lavage fluids of C3−/− mice versus wild-type mice at either 1 or 3 h following CLP (Table 1). Reconstitution of the C3-deficient mice with C3 protein not only restored filtration of neutrophils to levels similar to those observed in the wild-type mice (Figs 3b and 4f) but also resulted in enhanced clearance of bacteria (Table 1).

Clearly, not all of the mast cell degranulation (Fig. 2) or TNF-α production (Fig. 3a) in this model is complement-dependent and this may explain, at least in part, the neutrophil recruitment that occurs when CLP is performed in C3−/− mice (Fig. 3b). Multiple factors, in addition to products of the complement system, may contribute to mast cell activation and TNF-α production observed in CLP, including bacterial fimbrial adhesins18 and lipopolysaccharide (LPS)19.

Similarly, some of the important actions of complement in this model may occur independently of any effects of mast cells. Complement is thought to participate in host defence against bacterial infection at several stages, such as direct lysis of bacteria by the C5b-C9 membrane attack complex, opsonization of bacteria by C3b and iC3b ligands, and activation and chemotaxis of neutrophils through C3a and C5a peptides1. C3-deficient mice are highly sensitive to the toxic effects of systemic endotoxin14 and to infection with group B Streptococcus15. In addition, the serum of C3−/− mice fails to support bacteriolysis by neutrophils in an antibody-dependent opsonophagocytosis assay in vitro16. Thus, complement has some essential functions in bacterial clearance that are mast cell-independent.

C5 may have a role in the CLP model, both in assembly of the membrane attack complex on bacteria and because of the release of C5a anaphylatoxin, which can induce mast cell degranulation17 and function as an important chemo-attractant of neutrophils2. Indeed, comparison of mice that are genetically deficient in C5 (BL10/OSNJ)18 with controls (BL10/NSNJ) in the CLP model demonstrated a roughly 25-fold reduction in bacterial (E. coli) clearance at both 1 h (38.0 × 104 versus 1.3 × 105 CFU) and 3 h (55.6 × 104 versus 2.2 × 105 CFU), respectively, after overnight cultures of peritoneal lavage fluid pooled from five mice per group. By contrast, there was only a slight reduction in neutrophil infiltration in the C5-deficient strain relative to controls (40% versus 56% at 3 h, respectively (P = 0.230)). These findings, and work with C5ar−/− mice21, indicate that C5 can be important for efficient bacterial clearance, but may not be essential for the initial infiltration of neutrophils into the peritoneal cavity following bacterial infection.

Although the exact mechanism of complement activation in the CLP model is not yet clear, our results with C4-deficient mice indicate that the classic pathway is required (Fig. 1). It is possible that either or both of two important recognition molecules in natural immunity, natural antibody26,27 or mannan binding lectin28 (MBL) (each of which can lead to activation of the classic pathway) may be involved in complement activation in the CLP-model. It is possible that levels of natural antibody or MBL are different in C3-deficient mice versus the mast cell-deficient and congenic normal mice which were used in previous studies of CLP11, but the reconstitution of C3−/− mice with HuC3 largely repaired the defects in their response to CLP.

### Table 1 Bacterial clearance within the peritoneum is impaired in complement-deficient mice

<table>
<thead>
<tr>
<th>Mice</th>
<th>One hour after CLP</th>
<th>Three hours after CLP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CFU</td>
<td>CFU</td>
</tr>
<tr>
<td>WT</td>
<td>1.6 × 10⁵</td>
<td>5.7 × 10⁵</td>
</tr>
<tr>
<td>C3−/−</td>
<td>3.46 × 10⁵</td>
<td>1.16 × 10⁶</td>
</tr>
<tr>
<td>C3−/− plus 0.5mg HuC3</td>
<td>2.1 × 10⁵</td>
<td>3.42 × 10⁵</td>
</tr>
</tbody>
</table>

About 20-fold more colony-forming units (CFU) of E. coli were identified in the peritoneal lavage fluid of C3−/− mice compared to wild-type mice, at either 1 or 3 hours after CLP. But HuC3-reconstituted C3−/− mice exhibited only 6% or 30% of the number of CFU at 1 or 3 h after CLP than did the C3−/− mice that were not treated with HuC3. CFU were determined by overnight culture of serial dilutions of peritoneal lavage fluid which had been collected from five mice in each group and then pooled before culture in one experiment. In the second experiment, CFU represents the mean of individual mice with five mice per group.

* CFU of E. coli after overnight culture of peritoneal lavage fluid on LB-agar plates.
**A homologue of the TNF receptor and its ligand enhance T-cell growth and dendritic-cell function**


**Departments of Molecular Biology, Immunobiology and Bioinformatics, Immunex Corporation, 51 University St, Seattle, Washington 98101, USA**

Dendritic cells are rare haematopoietic cells that reside in a number of organs and tissues. By capturing, processing and presenting antigens to T cells, dendritic cells are essential for immune surveillance and the regulation of specific immunity. Several members of the tumour necrosis factor receptor (TNFR) superfamily are integral to the regulation of the immune response. These structurally related proteins modulate cellular functions ranging from proliferation and differentiation to

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Correspondence and requests for materials should be addressed to M.C.C. (e-mail: macroll@warren.med.harvard.edu).