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Cutting Edge: Targeted Disruption of the C3a Receptor Gene Demonstrates a Novel Protective Anti-Inflammatory Role for C3a in Endotoxin-Shock[1]

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The complement anaphylatoxin C3a, on binding the C3aR, mediates numerous proinflammatory activities. In addition, recent in vitro studies with C3a have implicated C3aR as a possible anti-inflammatory receptor. Because of its possible dual role in modulating the inflammatory response, it is uncertain whether C3aR contributes to the pathogenesis of endotoxin shock. Here, the targeted-disruption of the C3aR in mice is reported. These mice exhibit an enhanced lethality to endotoxin shock with a pronounced gene dosage effect. In addition, the plasma concentration of IL-1β was significantly elevated in the C3aR$^{-/-}$ mice compared with their littermates following LPS challenge. These findings demonstrate an important protective role for the C3aR in endotoxin shock and indicate that, in addition to its traditionally accepted functions in mediating inflammation, the C3aR also acts in vivo as an anti-inflammatory receptor by attenuating LPS-induced proinflammatory cytokine production. ***The Journal of Immunology,*** **2000, 165: 5406–5409.**

The C3a anaphylatoxin mediates numerous proinflammatory activities on binding its receptor, C3aR. These activities include release of lysosomal enzymes from leukocytes (1), secretion of histamine from mast cells (2), smooth muscle contraction (3), and chemoattraction of eosinophils (1) and mast cells (2, 4). Recent reports have suggested that C3a can also exhibit anti-inflammatory properties by suppressing LPS induced TNF-α, IL-1β, and IL-6 secretion from isolated PBMC (5, 6), and attenuating TNF-α and IL-6 secretion from B-cells stimulated with *Staphylococcus aureus* Cowan strain I and IL-2 (7).

Activation of complement during sepsis results in the generation of C3a and inactivated C3a (C3adesArg; Ref. 8). Several investigators have found a close relationship between elevated plasma levels of C3a/C3adesArg in patients with septic shock, and the risk of the development of adult respiratory distress syndrome or multisystem organ failure (9–12). Others, however, have not been able to demonstrate any positive correlation between these observations (13). Therefore, it has remained uncertain whether C3a contributes to the pathogenesis of septic shock.

To investigate the role of C3a in septic shock, C3aR$^{-/-}$ mice were produced by gene targeting and studied in an in vivo model of shock generated by i.v. injection of LPS. LPS challenge revealed increased susceptibility to shock in the C3aR$^{-/-}$ mice compared with their wild-type littermates. The increased sensitivity to LPS-induced shock was accompanied by significantly increased plasma concentrations of IL-1β and a trend toward increased plasma concentrations of TNF-α and IL-6 in the C3aR$^{-/-}$ mice as compared with wild-type mice. Collectively, these results indicate that the C3aR plays a significant role in tempering the pathological effects of endotoxin-induced shock and supports the in vitro data indicating that C3a functions as an anti-inflammatory molecule, as well as a proinflammatory mediator.

Materials and Methods

C3aR targeting vector and generation of C3aR null mice

A targeting vector was designed from a 129/Svj genomic clone (14) by replacing exon 2, containing the entire open reading frame of the C3aR gene, with the neomycin resistance gene from pKO SelectNeo V800 (Lexicon Genetics, The Woodlands, TX). RW-4 embryonic stem cells were transfected with the linearized targeting vector and the generation of chimeric and mutated mice was conducted as described previously (15).

Southern blot analysis

DNA was isolated from G418-resistant embryonic stem cells or from mouse tail biopsies and digested with *Hin*dIII. Standard Southern blotting was performed with a ^{32}P-radiolabeled 5′ probe external to the targeting vector.

PCR genotyping

DNA was isolated from mouse tail biopsies. The isolated DNA was used as a template in PCR with primers C1 (TACAATATAGTCAGTTGGAAGTCAGCC), NeoA (TGGGCTCTATGGCTTCTGAGGCGGAAAG), and A201$^+$ (GAGAATCAGGTGAGCCAAGGAGAAG). The primers C1 and NeoA yield a fragment of 537 bp for targeted DNA. Primers C1 and A201$^+$ yield a fragment of 726 bp for wild-type DNA.

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Antibodies

Polyclonal rabbit anti-mouse C3aR Abs were generated to a peptide corresponding to amino acid residues 240–258 of the mouse C3aR (CSPEDS FSLDSANQQPHYGG). By ELISA, it was determined that the rabbit antiserum raised specifically recognizes the C3aR peptide.

Immunofluorescence and flow cytometry

Thioglycollate-stimulated peritoneal macrophages were obtained from wild-type and C3aR$^{-/-}$ mice as described (16). Microscope slides containing 1.5×10^5 cells were prepared using a cytocentrifuge. After blocking nonspecific binding sites, the slides were incubated with rabbit anti-mouse C3aR antiserum diluted 1:1000. Bound Abs were detected with FITC-conjugated goat anti-rabbit F(ab′)$_2$ antiserum (Sigma, St. Louis, MO). Cells were analyzed on a FACScalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ).

Endotoxin induced shock and cell counts

Six to ten-wk old C3aR$^{-/-}$, C3aR$^{+/-}$, and wild-type littermates (on a C57BL/6 and 129SvJ mixed background) were injected (i.v.) with 48 mg/kg with LPS from *E. coli* serotype 0127:B8 (Sigma). The mice were injected at time 0 and monitored for survival at indicated times and at least twice daily for 7 days.

Cytokine and nitrate/nitrite measurements

Serum levels of TNF-α, IL-1β, IL-10, and IL-6 were determined using ELISA kits obtained from R&D Systems (Minneapolis, MN). Briefly, LPS-challenged mice were anesthetized and blood was collected by cardiac puncture. Plasma from three mice was pooled within each group. Three to four pools from each group were measured by ELISA. NO_3^- in duplicate samples of plasma was converted to NO_2^- with reductase (Sigma N-7265) and NADPH (ICN 101167). NO_2^- was quantified as described (17).

Statistical analysis

Statistical analysis was performed using the Graphpad (San Diego, CA) Prism program package. Survival curves were compared using the logrank test and ELISA results using the two-tailed, unpaired *t* test.

Results

Production and characterization of C3aR$^{-/-}$ mice

The mouse C3aR gene was disrupted by replacing the entire open reading frame, contained in exon 2, with a *neo^r* cassette (Fig. 1). To confirm that the gene disruption created a null mutation, immunofluorescence and flow cytometry of thioglycollate-stimulated peritoneal macrophages were used to demonstrate the absence of the C3aR in the C3aR$^{-/-}$ mice (Fig. 2). Furthermore, RT-PCR was performed using total RNA isolated from brain, kidney, liver, lung, testis, and thioglycollate-stimulated peritoneal macrophages of wild-type and C3aR$^{-/-}$ mice. RNA from wild-type mice yielded a PCR fragment corresponding to the C3aR, whereas RNA from homozygous knockout mice did not produce any fragment, confirming the absence of C3aR gene expression (data not shown).

Of 308 animals obtained from heterozygous matings, 26% were homozygous for the null allele. The newborn C3aR$^{-/-}$ mice developed normally in size and behavior and were fertile. Gross examination of several organs (brain, kidney, liver, lungs, testis, ovaries, heart, intestine, adrenal, and thymus) of C3aR$^{-/-}$ mice revealed no abnormalities. Furthermore, hematologic testing demonstrated normal cell populations in C3aR$^{-/-}$ and C3aR$^{+/-}$ mice compared with wild-type littermates (data not shown).

C3aR$^{-/-}$ mice display increased mortality to endotoxin-induced shock

To analyze whether the role of the C3aR serves dual functions (pro- and anti-inflammatory) in endotoxemia, 6- to 10-wk-old C3aR$^{-/-}$ mice and their wild-type littermates were injected i.v. with increasing doses of LPS. Following challenge, the C3aR$^{-/-}$ mice experienced increased susceptibility to the lethal effects of endotoxin compared with their wild-type littermates. At 48 mg/kg,

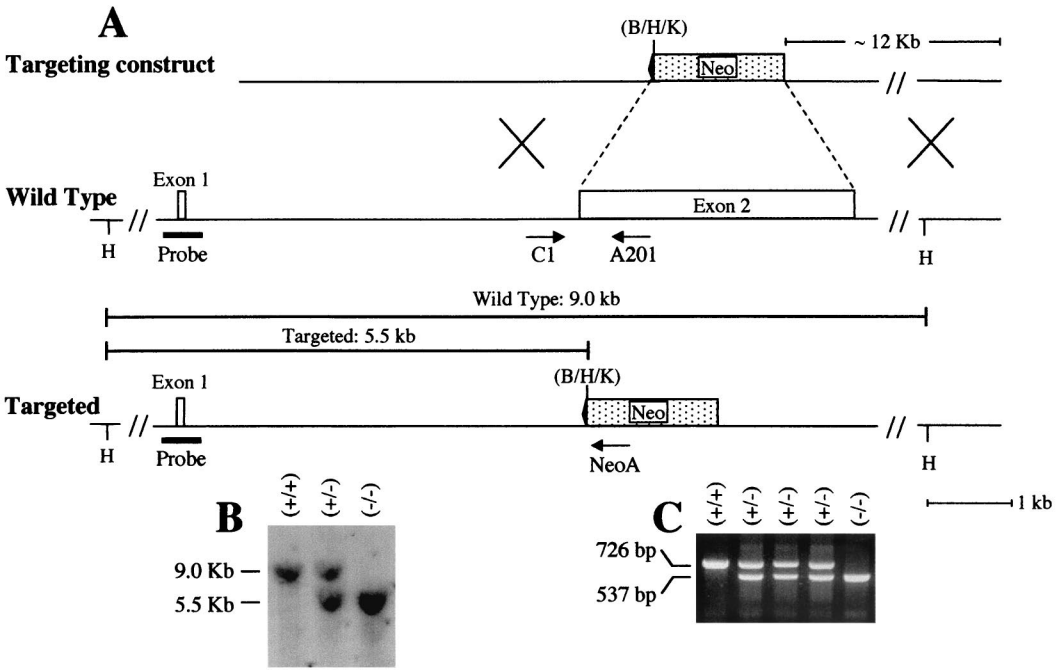

FIGURE 1. Targeting strategy, Southern blot analysis, and PCR genotyping. *A*, Schematic of the C3aR genomic locus, the targeting vector, and the targeted null locus. Exons are indicated by open boxes. B, *Bgl*II; H, *Hin*dIII; K, *Kpn*I. The position of the 5′ external probe is indicated. The arrows indicate the position of the oligonucleotides used for PCR genotyping. *B*, Southern blot analysis of *Hin*dIII-digested genomic DNA from mouse tails. Bands of 9.0 and 5.5 kb correspond to the wild-type and the targeted allele, respectively. Results from wild-type homozygous (+/+), C3aR-deficient heterozygous (+/−) and homozygous (−/−) mice are shown. *C*, PCR genotyping using the indicated oligonucleotides. For the wild-type and targeted alleles, fragments of 726 and 537 bp are generated, respectively.

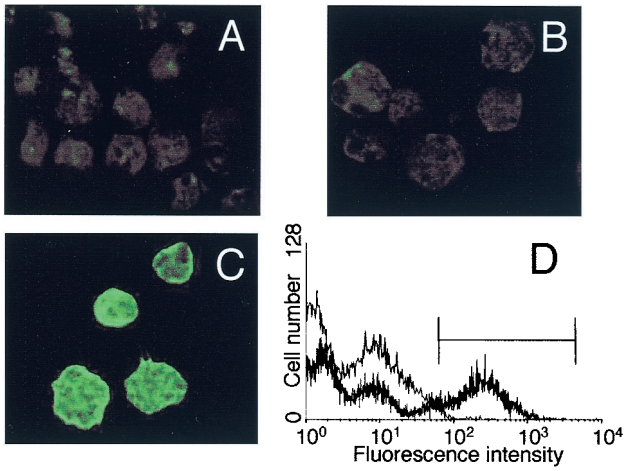

FIGURE 2. Immunofluorescence of thioglycollate-stimulated peritoneal macrophages demonstrating the absence of C3aR expression on cells isolated from C3aR$^{-/-}$ mice. *A*, Unstained macrophages from a C3aR$^{-/-}$ mouse indicating autofluorescence level. *B*, Macrophages from a C3aR$^{-/-}$ mouse stained with rabbit anti-mouse C3aR antiserum and FITC-conjugated anti-rabbit F(ab′)$_2$ antiserum. *C*, Macrophages from a wild-type mouse stained with rabbit anti-mouse C3aR antiserum and FITC-conjugated anti-rabbit F(ab′)$_2$ antiserum. *D*, Flow cytometry histogram of thioglycollate-stimulated peritoneal macrophages from C3aR$^{-/-}$ and wild-type mice. The cells were stained with rabbit anti-mouse C3aR antiserum and FITC-conjugated anti-rabbit F(ab′)$_2$ antiserum. The thin line represents C3aR$^{-/-}$ cells and the thick line represents wild-type cells. The gated cells correspond to 1.96% for the C3aR$^{-/-}$ cells and 33.9% for the wild-type cells.

85% of the C3aR$^{-/-}$ mice died within 3 days, compared with only 17% of the wild-type (Fig. 3). The C3aR heterozygous deficient mice (C3aR$^{+/-}$) were also more sensitive to LPS challenge than their wild-type littermates, but less sensitive than homozygous C3aR$^{-/-}$ mice, indicating a gene-dosage effect (Fig. 3).

C3aR$^{-/-}$ mice exhibit elevated levels of IL-1β after LPS challenge

To determine whether the increased sensitivity of the C3aR$^{-/-}$ mice to LPS-challenge is reflected in alterations of cytokine and NO levels, TNF-α, IL-1β, IL-6, IL-10, and nitrite (NO_2^-) and nitrate (NO_3^-) levels were determined in plasma samples obtained from C3aR$^{-/-}$ mice and their wild-type littermates injected with

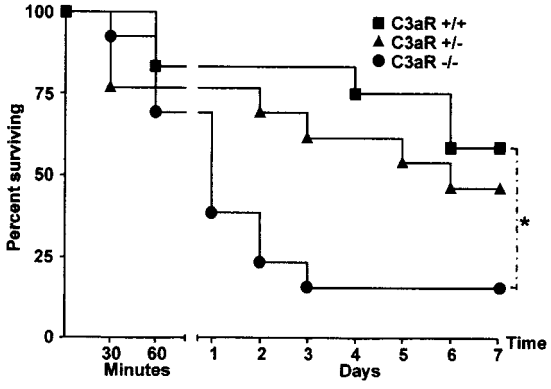

FIGURE 3. Survival curves for mice receiving 48 mg/kg LPS i.v. C3aR$^{-/-}$ mice are more sensitive to LPS-induced lethality. A gene dosage effect is apparent as mice heterozygous for the C3aR show intermediate sensitivity. $p = 0.0083$; $n = 12–13$.

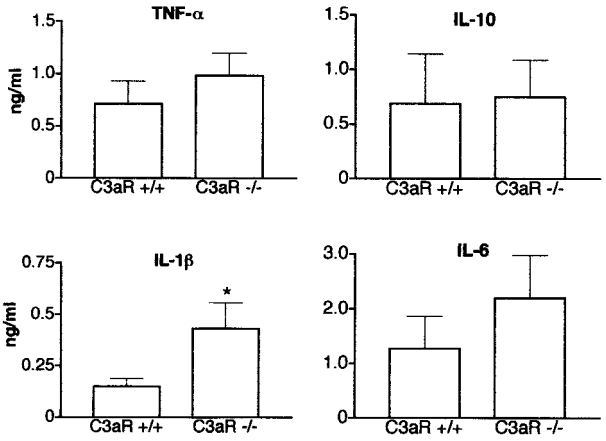

FIGURE 4. Plasma levels of IL-1β, TNF-α, IL-6, and IL-10 after LPS injection. The C3aR$^{-/-}$ mice have statistically significant elevated levels of IL-1β ($p = 0.046$). Results are expressed as mean ± SEM. Cytokines were not detectable before LPS injection.

LPS. Compared with their wild-type littermates, the C3aR$^{-/-}$ animals contained elevated plasma levels of all the proinflammatory cytokines (IL-1β, IL-6, and TNF-α) after LPS treatment (Fig. 4). IL-1β levels were significantly elevated, with an approximate 3-fold increase in the samples from C3aR$^{-/-}$ mice, 0.15 ng/ml ± 0.04 (SE) and 0.43 ng/ml ± 0.12 (SE) for wild-type and C3aR$^{-/-}$ mice, respectively. In contrast, there were no differences in the plasma levels of IL-10 and nitrite/nitrate in the C3aR$^{-/-}$ and wild-type mice after LPS challenge.

Discussion

This study has described the generation of C3aR$^{-/-}$ mice by homologous recombination, with a targeting vector that resulted in ablation of exon 2 of the C3aR gene. Mice homozygous for the C3aR null allele were completely deficient in the ability to express the C3aR, as shown by RT-PCR and immunofluorescence. The C3aR$^{-/-}$ mice appeared normal, were fertile, and contained no observable tissue or hematologic abnormalities. However, they were found to have increased susceptibility to the effects of LPS when subjected to an endotoxin-induced model of sepsis.

Measurements of IL-1β indicated a significant elevation in plasma concentration of this proinflammatory cytokine in the C3aR$^{-/-}$ mice, which may explain, at least in part, the increased LPS sensitivity. That high levels of IL-1β may be a major contributor to the increased LPS sensitivity is supported by the observation that deficiency of the IL-1β converting enzyme protects mice from LPS-induced shock (18).

The increased sensitivity of C3aR$^{-/-}$ mice to i.v. injection of LPS might be considered paradoxical, because the C3a ligand is generally viewed as a mediator of proinflammatory functions. However, recent reports have demonstrated in vitro that C3a may act as an anti-inflammatory molecule by suppressing LPS induced secretion of TNF-α, IL-1β, and IL-6 from isolated PBMC (5, 6), as well as TNF-α and IL-1β production from *S. aureus* Cowan strain I/IL-2-stimulated B-cells (7). Our observations of increased mortality and elevated IL-1β plasma concentrations in LPS-treated C3aR$^{-/-}$ mice support these in vitro data. Moreover, they suggest that the anti-inflammatory effects of C3a are significant in vivo, and can occur through specific interactions with the C3aR.

The genetically C3aR$^{-/-}$ mice will be useful models to further investigate the yet unknown functions of C3a, and its participation

in numerous diseases in which complement anaphylatoxin peptides have been implicated, including asthma, myocardial ischemia injury, rheumatoid arthritis, inflammatory bowel disease, and psoriasis.

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