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Deletion of Both the C3a and C5a Receptors Fails to Protect Against Experimental Autoimmune Encephalomyelitis

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Abstract

Multiple sclerosis (MS) is an autoimmune disease in which inflammation, leukocyte infiltration, and ultimately, demyelination occur as a result of innate and adaptive immune-mediated mechanisms. The pathophysiological role of the complement system, a major component of innate immunity, in the development and progression of experimental autoimmune encephalomyelitis (EAE), the animal model for MS has been extensively examined. Previous studies from our lab have shown that the complement receptor for the anaphylatoxin C3a, but not for C5a plays an important role in EAE. Based on the important contributions of the complement anaphylatoxin receptors to other inflammatory conditions in the CNS, we reasoned that deletion of both receptors may reveal underlying interactions between them that are important to EAE pathology. We performed EAE in C3aR/C5aR double knockout mice (C3aR/C5aR^{-/-}) and observed delayed onset of disease but no attenuation of disease severity compared to wild type mice. Interestingly there was trend toward greater infiltration of CD4⁺, but not CD8⁺ T cells, in C3aR/C5aR^{-/-} mice with EAE, suggesting altered trafficking of these cells. Antigen-specific T cells isolated from C3aR/C5aR^{-/-} mice during acute EAE produced elevated levels of TNF- α , but markedly reduced levels of IFN- γ and IL-12 compared to wild type mice. It remains unclear how the changes in these disease parameters contribute to the loss of the protective effect seen in C3aR^{-/-} mice, however our data indicate a level of cross modulation between the C3aR and C5aR during EAE.

Keywords

experimental autoimmune encephalomyelitis; anaphylatoxin receptors; complement; neuroimmunology

The role of the complement system in the development of experimental autoimmune encephalomyelitis (EAE), the animal model for multiple sclerosis has been studied in detail (reviewed in [1]). Activation of complement results in the production of C3a and C5a, two pro-inflammatory and chemoattractant polypeptides that mediate many of the inflammatory functions of the complement system [7]. Several studies have shown, however, that the canonical C5a receptor is not critical to the development and progression of EAE [8–10]. In contrast, deletion of the C3aR provides some protection from disease, while C3a expressed in the CNS under the control of a GFAP promoter exacerbates disease, often fatally so [2]. Based

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on these studies and the numerous contributions they make to inflammatory responses in and out of the CNS, we reasoned that there may be redundant functions for the C3aR and C5aR in the pathogenesis of demyelinating disease. To address this possibility, we examined EAE in C3aR/C5aR double knockout mice.

For these studies C3aR^{-/-} [6] and C5aR^{-/-} mutant mice [5] were crossed. All mice were previously backcrossed to the C57BL/6 background for greater than eight generations and inbred C57BL/6 mice were used as controls. We induced EAE in wild type and C3aR/C5aR^{-/-} mice as previously described [11]. Onset and progression of EAE symptoms was monitored daily using a standard clinical scale ranging from 0 to 6 as follows: 0, asymptomatic; 1, weak tail; 2, flaccid tail; 3, incomplete paralysis of one or two hind limbs; 4, complete hind limb paralysis; 5, moribund; 6, dead. Only mice with a score of at least 2 (flaccid tail) for more than 2 consecutive days were judged to have onset of EAE. For each animal a cumulative disease index (CDI) was calculated from the sum of the daily clinical scores observed through day 30. All animal procedures were performed with approval from the UAB IACUC.

For isolation of leukocytes and flow cytometry, mice were perfused and spinal cords were prepared and stained as previously described [11]. Cell preparations were incubated with anti-CD16/32 (FcR block, eBioscience) to prevent nonspecific staining. Cells were then stained at 4°C with PE conjugated anti-CD4 (GK-1.5 eBioscience), FITC conjugated anti-CD3 (145-2C11; eBioscience), and APC conjugated anti-CD8 (53-6.7; eBioscience). Immunofluorescence analyses were performed using a FACS Calibur and CellQuest software (BD Biosciences). For proliferation assays, lymph nodes were taken from mice with active EAE on day 8 and single cell preparations were cultured for 48 hours with increasing concentrations of MOG₃₅₋₅₅ peptide (1.5 to 6 µg/ml). [³H] thymidine was added and 18 hours later cells were harvested and measured for thymidine incorporation. For determination of T cell-produced cytokines, lymph nodes were taken from mice with active EAE on day 9, and cultured for 72 hours with MOG₃₅₋₅₅ peptide (20µg/ml). Supernatants were collected and analyzed by ELISA using kits for IFN-γ, TNF-α, IL-12 and IL-17 according to the manufacturers instructions (R&D Systems).

We compared the course of active EAE between wild type and C3aR/C5aR^{-/-} mice and observed that the onset of disease in C3aR/C5aR^{-/-} mice was significantly delayed compared to that of wild type mice (Fig. 1, Table 1; 12.2d vs. 14.2d, p=0.046, Student's t-test). Despite the difference in this disease parameter, the progression of acute and chronic disease was essentially identical between the two groups of mice and the cumulative disease index was not significantly different (p>0.05, Student's t-test). The severity of EAE in C3aR/C5aR^{-/-} mice was somewhat unexpected since we have previously shown that the chronic phase of EAE in C3aR^{-/-} mice is significantly attenuated [2]. We anticipated a recapitulation of the C3aR^{-/-} phenotype particularly since C3aR^{-/-} × C3a/GFAP mice were also significantly protected from disease, indicating an important role for the C3aR in the pathogenesis of EAE. It remains unclear how the loss of the C5aR compromises the protective effect of C3aR deletion in EAE. It may be that, despite its own redundant role in EAE, the C5aR modulates some aspect of C3aR intracellular signaling or expression on one or more cell types during the course of disease.

We also examined the composition of the cellular infiltrate and cytokine production by T cells between the two groups of mice. The overall percentage of T cells in the spinal cord of the C3aR/C5aR^{-/-} mice was not significantly different than that seen in wild type mice (data not shown). In contrast, we observed differences between CD4⁺ T cell infiltration into spinal cords of the anaphylatoxin mutant mice, but not for CD8⁺ T cells. Approximately three-fold more CD4⁺ T cells were present in the spinal cords of C3aR/C5aR^{-/-} mice at day 15 post-induction compared to wild type mice, however the difference was not significant (Fig. 2). Despite the

elevated CNS infiltration, we found no difference in antigen-specific T cell proliferation in response to MOG peptide, between the two groups suggesting that the loss of both receptors did not impair their ability to respond to antigen (data not shown). Interestingly, T cells from lymph nodes of C3aR/C5aR^{-/-} mice with EAE produced an unusual profile of cytokines compared to wild type mice with elevated TNF- α (~5-fold, not significant) but reduced IFN- γ (~3-fold, $p < 0.05$, Student's t-test), IL-12 (~4-fold, not significant) and IL-17 (essentially identical) (Fig. 3). The lack of combined anaphylatoxins receptor-mediated signaling in one or more cell types may alter the expression of cytokines and chemokines that are critical to the development and progression of EAE and thus contribute the disease phenotype we observed in C3aR/C5aR^{-/-} mice.

The phenotype of EAE we observed in C3aR/C5aR^{-/-} mice was unexpected given the general importance of these receptors to inflammatory events in many autoimmune disease settings [4,7]. Our results suggest that unanticipated mechanistic inter-relationships occur between the C3a and C5a receptors and that their contribution to autoimmune disease pathogenesis (or lack thereof) may be very disease specific. Additional studies using C3aR/C5aR^{-/-} mice need to be performed in other disease models to verify this possibility. Our results support previous observations in ICAM-1^{-/-} $\times$ C3^{-/-} mice [12] in that the protective features associated with one genotype in EAE can be overcome in the double mutant genotype. In this case the attenuated EAE reported for C3aR^{-/-} mice [2] was lost in C3aR/C5aR^{-/-} mice. A logical extension of these studies is to examine the role of the C5aL receptor in EAE using C5aL^{-/-} mice [3] and ultimately to cross these mice with canonical receptor-deficient mice. From an anaphylatoxin receptor-centric point of view, such studies could provide additional mechanistic insight. Regardless, these results combined with previous studies on the C3a and C5a receptors [2,8–10], clearly indicate that these receptors are unlikely targets for therapeutic intervention in demyelinating disease.

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Figure 1.

Onset of active EAE is delayed in C3aR/C5aR^{-/-} mice. EAE was induced with MOG₃₅₋₅₅ peptide and signs of disease scored for 30 days as described previously. Results shown are the daily mean clinical scores ($n \pm \text{SEM}$) for wild type ($n=10$), C3aR/C5aR^{-/-} ($n=18$) from two pooled experiments.

Figure 2.

CD4⁺ T cell infiltration into the spinal cord is increased in C3aR/C5aR^{-/-} mice during EAE. Leukocytes isolated from spinal cords of control (n=3) and C3aR/C5aR^{-/-} mice (n=3) as described, were immunostained for CD3, CD4, and CD8. At day 15 post-immunization CD4⁺ T cell but not CD8⁺ T cell infiltration was increased in C3aR/C5aR^{-/-} mice compared to controls. The results shown are from a representative mouse from each group.

Figure 3.

Encephalitogenic C3aR/C5aR^{-/-} T cells produce high levels of TNF- α , reduced levels of IFN- γ and IL-12 and unchanged levels of IL-17. Cells from lymph nodes of wild type and C3aR/C5aR^{-/-} mice with EAE were isolated and stimulated with MOG₃₅₋₅₅ peptide (20 μ g/ml) for 72 hours. Cytokines were measured in culture supernatants using ELISA. Results shown are mean $\pm$ SE for four independent experiments. *, $p < 0.05$, student's t-test.

Table 1Active EAE signs in wild type and C3aR/C5aR^{-/-} mice.

	CDI ^A	Disease Onset ^B	Disease Incidence ^C	Mortality
WT (n=10)	50.2	12.2d	100	10%
C3aR/C5aR ^{-/-} (n=18)	44.2	14.2d*	100	6%

^A Cumulative Disease Index is the mean sum of daily clinical scores observed between days 7 and 30.

^B Disease onset is defined as the first day of two consecutive days with a clinical score of two or more.

^C Disease incidence is defined as the percent of mice that displayed any clinical signs of disease.

* Onset of EAE was significantly different as determined by Student's t-test, p=0.046.