Published in final edited form as:

Neurosci Lett. 2008 September 12; 442(2): 158–160. doi:10.1016/j.neulet.2008.07.005.

Deletion of both ICAM-1 and C3 Enhances Severity of Experimental Autoimmune Encephalomyelitis Compared to C3-Deficient Mice

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Abstract

Multiple sclerosis (MS) is an autoimmune disease characterized by central nervous system (CNS) inflammation and leukocyte infiltration, demyelination of neurons, and blood-brain barrier breakdown. The development of experimental autoimmune encephalomyelitis (EAE), the animal model for MS is dependent on a number of components of the immune system including complement and adhesion molecules. Previous studies in our lab have examined the role of C3, the central complement component, and intercellular adhesion molecule-1 (ICAM-1) a key cell adhesion molecule involved in leukocyte trafficking to sites of inflammation including the CNS. In these studies we demonstrated that myelin oligodendrocyte glycoprotein (MOG)-induced EAE is markedly attenuated in both ICAM-1^{-/-} and C3^{-/-} mice. Given the pivotal role that these proteins play in EAE, we hypothesized that EAE in ICAM-1^{-/-} and C3^{-/-} double mutant mice would likely fail to develop. Unexpectedly, EAE in ICAM-1^{-/-} × C3^{-/-} mice was only modestly attenuated compared to wild type mice and significantly worse than C3^{-/-} mice. Leukocyte infiltration was commensurate with disease severity between the three groups of mice. Spinal cord T cells from ICAM-1^{-/-} × C3^{-/-} mice produced the highest levels of IFN-γ and TNF-α, despite reduced disease severity compared to wild type mice. The mechanisms behind the elevated EAE severity in ICAM-1^{-/-} × C3^{-/-} mice may relate to altered homing of leukocytes or processing of self-antigens in the double mutant background.

Keywords

experimental autoimmune encephalomyelitis; adhesion molecules; complement; neuroimmunology

The role of complement and adhesion molecules in the development of experimental autoimmune encephalomyelitis (EAE), the animal model for multiple sclerosis has been extensively studied [1,2,11]. In the course of these studies it has become clear that two ligands for members of the β₂-integrin family of adhesion molecules, C3 and ICAM-1, play a critical

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role in the onset and progression of EAE. Null mutations in either gene significantly delay the onset of, and reduce the severity of EAE [6,14]. Likewise, the deletion of the β_2 -integrin receptors that bind C3 and ICAM-1, namely Mac-1 and p150,95, also significantly attenuates EAE [4,5]. These studies indicate important and nonredundant functions for these proteins in the pathogenesis of demyelinating disease. Based on the significant, but incomplete protection from EAE observed when using mice deficient in either protein, we reasoned that a double mutant would develop little to no EAE, or at the very least, EAE comparable to that of either single mutant. To address possible complement and adhesion molecule mechanistic inter-relationships, we examined EAE in ICAM-1 $\times$ C3 double knockout mice.

For these studies we crossed ICAM-1^{-/-} [6] with C3^{-/-} mutant mice [10]. All mice had been 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, ICAM-1^{-/-} $\times$ C3^{-/-} and C3^{-/-} mice as previously described [13]. Onset and progression of EAE symptoms was monitored daily using a standard clinical scale ranging from 0 to 5 as follows: 0, asymptomatic; 1, flaccid tail; 2, incomplete paralysis of one or two hind limbs; 3, complete hind limb paralysis; 4, moribund; 5, dead. Only mice with a score of at least 1 (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 lymph nodes, spleen and spinal cord were prepared and stained as previously described [13]. For surface staining, cells were incubated with anti-CD16/32 (FcR block, eBioscience) to prevent nonspecific staining. Cells were then stained at 4°C with PE conjugated anti-CD45 or FITC conjugated anti-CD45 (30-F11; eBioscience), APC conjugated anti-CD8 (Ly-2; eBioscience), Biotin conjugated anti-CD4 or FITC conjugated anti-CD4 (GK-1.5 eBioscience). Biotin conjugated staining was visualized with Streptavidin-PerCP (BD Pharmingen). All antibodies and secondary reagents were diluted in FACS buffer (1 $\times$ PBS, 2% FCS, 0.1% NaN₃). For intracellular cytokine staining, cells were first stained for surface receptor expression as described above, then incubated with 1 $\times$ fixation/permeabilization solution as recommended by the manufacturer (BD Pharmingen). Cells were washed and incubated with PE conjugated anti-TNF- α (MP6-XT22, eBioscience) and FITC conjugated anti-IFN- γ (XMG1.2, eBioscience) or PE conjugated anti-IL-4 (11B11, eBioscience) and FITC conjugated anti-IL-10 (JESS-16E3, eBioscience) in 1 $\times$ permeabilization solution for 45 minutes in the dark at room temperature. Four-color immunofluorescence analyses were performed using a FACS Calibur and CellQuest software (BD Biosciences). Proliferation assays were performed using the Cell Titer 96 AQueous One Solution Cell Proliferation Assay (Promega) according to manufacturer's instructions. Briefly, after 72 hours of anti-CD3 stimulation, 40 μ l of MTS was added to cultures (10 μ l/100 μ l media). Cultures were then incubated for 3 hours and absorbance was measured at 450nm.

We compared the course of active EAE between wild type, ICAM-1^{-/-} $\times$ C3^{-/-} and C3^{-/-} mice and observed that the onset and initial course of disease in ICAM-1^{-/-} $\times$ C3^{-/-} mice was remarkably similar to that of wild type mice (Fig. 1, Table 1). The chronic phase of disease in ICAM-1^{-/-} $\times$ C3^{-/-} mice was attenuated compared to wild type mice and, as a result, the overall disease severity between the two groups of mice was significantly different ($p < 0.01$, 1-way ANOVA, Newman-Keuls multiple comparisons post hoc test). In contrast, ICAM-1^{-/-} $\times$ C3^{-/-} mice developed EAE much sooner than C3^{-/-} mice (day 18.3 vs. day 25.4) and both the initial and overall course of disease were significantly worse ($p < 0.05$, 1-way ANOVA, Newman-Keuls multiple comparisons post hoc test). The severity of EAE in ICAM-1^{-/-} $\times$ C3^{-/-} mice was unexpected since both C3^{-/-} and ICAM-1^{-/-} mice have markedly delayed and attenuated EAE [6,14].

We also examined the composition of the cellular infiltrate and cytokine production by T cells between the three groups of mice. The overall percentage of CD45⁺ cells into the spinal cord, as a marker of leukocyte infiltration, was highest in wild type mice and lowest in C3^{-/-} mice (wild type: 94%, ICAM-1^{-/-} × C3^{-/-}: 81%, C3^{-/-}: 72%), a pattern that mimics the overall severity of EAE. Interestingly, ICAM-1^{-/-} × C3^{-/-} mice had reduced numbers of both CD4⁺ (7.3% vs. 19.7%) and CD8⁺ (6.8% vs. 14.5%) T cells in the spinal cord at the peak of disease (day 15) compared to wild type mice (Fig. 2). These results suggest that the overall diminution of leukocytes in the CNS of ICAM-1^{-/-} × C3^{-/-} mice at this point in the disease course is attributable largely to reduced T cell infiltration. Surprisingly a higher percentage of both CD4⁺ and CD8⁺ T cells derived from ICAM-1^{-/-} × C3^{-/-} mice produced TNF-α and IFN-γ at day 15 post-immunization compared to wild type and C3^{-/-} mice (Table 2). These findings suggest that a combination of reduced T cell trafficking and the potential anti-inflammatory effects of IFN-γ and TNF-α in this setting, may contribute to the attenuated EAE observed in ICAM-1^{-/-} × C3^{-/-} mice.

Compared to wild type mice, the profile of EAE *in vitro* parameters (Table 1) we observed for ICAM-1^{-/-} × C3^{-/-} mice were consistent with the moderately attenuated disease course, but do not explain why the double mutant mice have worse compared to C3^{-/-} mice. Interestingly, T cells from ICAM-1^{-/-} × C3^{-/-} mice proliferate comparably to wild type T cells (Fig. 3) in antigen-specific assays, a finding similar to that observed in C3^{-/-} mice [14], but not in ICAM-1^{-/-} mice [6]. However, T cells from ICAM-1^{-/-} × C3^{-/-} mice did not proliferate as well as wild type T cells when stimulated with anti-CD3 (Fig. 3). Thus, in some settings the protective features associated with one genotype in EAE can be overcome in the double mutant genotype, but not in others.

We cannot rule out that the disease phenotype observed in ICAM-1^{-/-} × C3^{-/-} mice is due to altered homing mechanisms allowing for greater leukocyte infiltration and subsequent increased histopathology compared to single mutant mice. Efforts are underway to address this possibility. Based on what is known for the role of complement in autoimmune disease (reviewed in [3]), it may be that the loss of both C3 and ICAM-1 alters the handling of apoptotic cells in such a way that it elicits a stronger autoimmune response than that seen with the loss of either gene alone. In this scenario, apoptotic bodies, which contain high concentrations of self-antigens, may generate altered antigens by unusual cleavage or phosphorylation during the apoptotic process [7–9,12,15]. As a result, modified self-antigen may expose cryptic epitopes that lead to a break in tolerance (possibly enhanced epitope spreading) and significantly worse EAE in ICAM-1^{-/-} × C3^{-/-} mice than either single mutation. Our studies demonstrate that despite well-established roles for this receptor/ligand pair in EAE, the extent of their interactions in contributing to the development of demyelinating disease remains unclear.

Acknowledgements

This work was supported by NIH grants NS46032 to SRB and DCB and, T32 AI07051 to SSS and JEW.

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Figure 1.

Clinical course of actively induced EAE is exacerbated in ICAM-1^{-/-} x C3^{-/-} mice compared to C3^{-/-} mice. EAE was induced with MOG₃₅₋₅₅ peptide and signs of disease scored for 30 days as described in Materials and Methods. Results shown are the daily mean clinical scores ($n \pm \text{SEM}$) for wild type ($n=16$), ICAM-1^{-/-} x C3^{-/-} ($n=23$) and C3^{-/-} ($n=22$) from two pooled experiments.

Figure 2.

Leukocyte subset infiltration in the spinal cord of $ICAM-1^{-/-} \times C3^{-/-}$ mice with EAE is reduced compared to control mice. Leukocytes isolated from spinal cords of control (n=4) and $ICAM-1^{-/-} \times C3^{-/-}$ mice (n=4) as described were immunostained for CD4 and CD8. At day 15 post-immunization CD4 and CD8 infiltration was reduced in $ICAM-1^{-/-} \times C3^{-/-}$ mice compared to controls. The results shown are from cells pooled within each group of mice.

Figure 3.

Encephalitogenic ICAM-1^{-/-} × C3^{-/-} T cells proliferate comparably to control T cells. Cells from spleen and lymph nodes of wild type and ICAM-1^{-/-} × C3^{-/-} mice with EAE were isolated and stimulated with increasing concentrations of MOG peptide (0–25 µg/ml) or immobilized anti-CD3 antibodies for 72 hours. Proliferation was assessed using an MTS assay system as described in the text. Results shown are mean ± SE for four independent experiments. *, $p < 0.05$, student's t-test.

Table 1

Transferred EAE signs in wild type, ICAM-1 $\times$ C3^{-/-} and C3^{-/-} mice.

	CDI ^A	Disease Onset ^B	Disease Incidence ^C	% Mortality
WT (n=16)	47	14.6d	100	0
ICAM-1 $\times$ C3 ^{-/-} ^D (n=23)	28	18.3d	100	0
C3 ^{-/-} (n=22)	12	28.3d	78	0

^A Cumulative Disease Index is the mean of the 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.

^D EAE was significantly different between all three groups of mice as determined by one-way ANOVA ($p < 0.0001$) and by Newman-Keuls multiple comparisons test: WT vs. ICAM-1 $\times$ C3^{-/-} ($p < 0.01$), WT vs. C3^{-/-} ($p < 0.001$) and ICAM-1 $\times$ C3^{-/-} vs. C3^{-/-} ($p = 0.05$).

Table 2

Cytokine production by CD4 and CD8 T cells derived from spinal cords of wild type, ICAM-1 $\times$ C3^{-/-} and C3^{-/-} mice during EAE.

Genotype	CD4 ⁺ T cells ^A		CD8 ⁺ T cells	
	TNF- α	IFN- γ	TNF- α	IFN- γ
Wild type	26 $\pm$ 4	0	3.1 $\pm$ 0.8	1.3 $\pm$ 1.8
ICAM-1 $\times$ C3 ^{-/-}	38 $\pm$ 4	0	19 $\pm$ 7	1.2 $\pm$ 2
C3 ^{-/-}	21	2.5	4	0

^A Mean percent cytokine positive cells derived from mice at day 15 post-immunization. Cells were pooled from at least 2 mice per group from 2 independent experiments.