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Semliki Forest virus infection leads to increased expression of adhesion molecules on splenic T-cells and on brain vascular endothelium

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Semliki Forest virus A7 (SFV-A7) is a neurotropic alphavirus that leads to an asymptomatic encephalitis in adult immunocompetent mice. We studied the expression of leukocyte and endothelial cell adhesion molecules in the spleen and in the central nervous system (CNS) during SFV-A7 infection. Kinetics of the expression of LFA-1α/CD11a, LFA-1β/CD18, Mac-1/CD11b, VLA-4/CD49d, ICAM-1/CD54 and L-selectin/CD62L was determined on splenic CD4$^+$ and CD8$^+$ T-cells and macrophages by flow cytometry. Time course of the expression of these antigens and VCAM-1/CD106 as well as viral antigens in the CNS was studied by immunoperoxidase staining. In the spleen, a sustained increase in LFA-1-expression and a temporary increase at day 7 in the expression of VLA-4, Mac-1 and ICAM-1 were detected on CD8$^+$ T-cells. L-selectin was down-regulated on CD4$^+$ cells. Adhesion molecules on macrophages remained unchanged. In the CNS, expression of Mac-1$^+$, VLA-4$^+$ and LFA-1$^+$ cells increased in parallel with the kinetics of the expression of their ligands ICAM-1 and VCAM-1 on brain vessels. Upregulation of adhesion molecules peaked between days 5–8 and was most prominent in the cerebellar and brain stem white matter where viral antigens were most abundant. We conclude that the adhesion molecules profile of splenic T cells is altered during SFV-A7 infection which may influence their homing into the CNS. Macrophages are probably recruited non-specifically as a consequence of activation of the brain vascular endothelium in the inflamed areas of the brain.

Keywords: Semliki Forest virus; adhesion molecules; vascular endothelium; multiple sclerosis

Introduction

Semliki Forest virus (SFV) is a member of the family *Togaviridae*. Infection of immunocompetent adult mice with an attenuated mutant SFV-A7 leads to a nonlethal encephalitis and lesions of immune-mediated demyelination (Berger, 1980, Fazakerley and Webb, 1987; Suckling *et al*, 1978). CD4$^+$ and CD8$^+$ T-cells and macrophages have been identified in the central nervous system (CNS) mononuclear cell infiltrates during SFV-A7 infection (Erälinna *et al*, 1994). Detailed studies focusing on the mechanisms of demyelination during SPV-A7 infection have shown that antibodies clear the viremia and CD8$^+$ T cells are necessary for the development of lesions of demyelination (Amor *et al*, 1996; Subak-Sharpe *et al*, 1993).

The mechanisms leading to accumulation of mononuclear cells into the CNS during SFV-A7 infection are largely undefined. The emigration of leukocytes from the bloodstream into the tissues is a multistep process involving interaction of different families of adhesion receptors on the surface of leukocytes with their specific ligands on the vascular endothelial cells (EC) (Butcher, 1991). In inflammation, dynamic changes occur on the vascular endothelium, which direct leukocyte traffic to the site of immunologic challenge (Osborn, 1990). In the brain, vital encephalitis and meningitis have been shown to lead to upregulation of adhesion molecules on cerebral blood vessels (Sobel, 1990; Marker, 1995). Changes may also occur in the expression of adhesion receptors on the circulating leukocytes which favour their

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localization into tissues instead of lymph nodes (Springer, 1994).

We have earlier shown that SFV-A7 infects mouse brain endothelial cells and leads to blood–brain barrier (BBB) damage and increased expression of ICAM-1 (intercellular adhesion molecule-1) in the brain (Soilu-Hanninen *et al.*, 1994). This may facilitate the entry of inflammatory cells into the inflamed CNS, since leakage of the BBB diminishes the shear force provided by blood moving parallel to the EC surface and may thus favour leukocyte adherence to the EC (Prober and Cotran, 1990). Moreover, *in vitro* work has shown that lymphocyte transendothelial migration in the CNS is mostly supported by LFA-1 pairing with ICAM-1 (Greenwood *et al*, 1995). However, *in vivo* studies have suggested that ICAM-1 is not critically involved in autoimmune or cytokine-induced inflammation of the CNS (Willenborg *et al*, 1993; Barten and Ruddle, 1994). Therefore it was important to study whether other adhesion-molecule ligand pairs could be involved in leukocyte entry into the CNS during SFV-A7 infection.

We studied the expression of a bettery of leukocyte and endothelial cell adhesion molecules on splenic $CD4^+$ and $CD8^+$ cells and macrophages and in the CNS after intraperitoneal infection of mice with SFV-A7. We also compared the kinetics and distribution of virus antigen expression to the changes in adhesion molecule expression in the CNS. The most marked change in the spleen was a sustained increase in the expression of LFA-1 on $CD8^+$ cells. A temporary increase in the expressions of VLA-4, Mac-1 (type 3 complement receptor) and ICAM-1 on $CD8^+$ cells as well as downregulation of L-selectin on $CD4^+$ cells were also observed. Adhesion molecules on splenic macrophages were unaltered. In the CNS, expressions of ICAM-1 and VCAM-1 (vascular cell adhesion molecule-1) were increased on blood vessels peaking between days 5 and 8 p.i. corresponding to the infiltration of inflammatory cells expressing their integrin ligands LFA-1 and VLA-4. Activation of the CNS endothelium was most prominent in the cerebellar and brain stem white matter where viral antigens were most abundantly expressed. *In vivo* treatment with mAbs against ICAM-1 and VCAM-1 decreased the expression of LFA-1 and VLA-4 in the CNS.

Taken together our results suggest that changes in the adhesion molecule profile of T cells at the site of the primary immune response during SFV-A7 infection may influence their subsequent homing into the brain. Macrophages are probably recruited non-specifically due to upregulation of adhesion molecules on the vascular endothelium in the inflamed areas of the brain.

Results

Expression of adhesion molecules on splenic CD4 and CD8 T cells and macrophages during SFV-A7 infection

We studied if infection of mice with SFV-A7 leads to an altered pattern of adhesion molecule expression on the splenic leukocyte subpopulations, that could influence their homing into the brain. Therefore we analyzed by flow cytometry the expression of a battery of adhesion molecules on spleen $CD4^+$ and $CD8^+$ T-cells and macrophages at different timepoints after intraperitoneal (i.p.) infection of mice with 1×10^6 PFU of SFV A7. We first determined the percentage of cells positive both for the leukocyte subtype marker and each adhesion molecule marker in all spleen cells during the course of the infection. The most marked change was a sustained increase in the percentage of LFA-1-positive CD8 cells in all spleen cells (Figure 1a). A temporary increase in the proportion of VLA-4-positive, Mac-1-positive and ICAM-1-positive $CD8^+$ cells at day 7 after infection was also observed as well as a decrease in the number of L-selectin$^+$ CD4 cells between days 3 and 11 p.i. (Figure 1a and b). Number of macrophages positive for the adhesion molecules studied of all spleen cells remained low, around 5% until day 24, when a 2–3-fold increase in the number of macrophages positive for each antigen was observed (Figure 1c). This was caused by an increase in the percentage of macrophages of all spleen cells from 4–15% by day 24 p.i., whereas percentages of CD4 and CD8 cells remained the same (data not shown).

To directly compare the amount of each adhesion molecule expressed on the surface of leukocytes obtained from infected mice with that of control mice, mean fluorescence intensity (MFI) values for adhesion molecule expression were determined for cells falling within the gates set for $CD8^+$ cells, $CD4^+$ cells and macrophages. The expressions of LFA-1α and LFA-1β, ICAM-1, VLA-4 and Mac-1 were increased on $CD8^+$ cells (Figure 2a) while expression of L-selectin was downregulated on $CD4^+$ cells (Figure 2b).

Expression of adhesion molecules and viral antigens in the CNS during SFV-A7 infection

Changes in the expression of adhesion molecules in the spleen is relevant for the pathogenesis of the CNS disease if these changes are reflected in the brain. We therefore immunostained frozen sections of brain and spinal cord tissue of SFV-A7 infected BALB/c mice for the expression of LFA-1α, Mac-1 and their ligand ICAM-1 and VLA-4 and its ligand VCAM-1. Results from the two infected mice that were studied at each timepoint as well as to

uninfected, age and sex-matched controls are shown in Figure 3. VLA-4 and LFA-1α were not expressed at detectable levels in the CNS of the healthy control mice (Figure 3; Figure 4c and e), and expression of ICAM-1 was low or undetectable (Figure 4). A few scattered Mac-1 positive cells were detected in the white matter probably representing the resident microglial cell population (Figure 3). Weak immunoreactivity for VCAM-1 was detectable diffusely on vascular endothelial cells but not in the CNS parenhyma. (Figure 3; Figure 4a).

At day 1 after infection, expression of LFA-1 was still undetectable and expression of Mac-1 and ICAM-1 was at a similar low level as in controls, but a few VLA-4-positive cells were detectable in

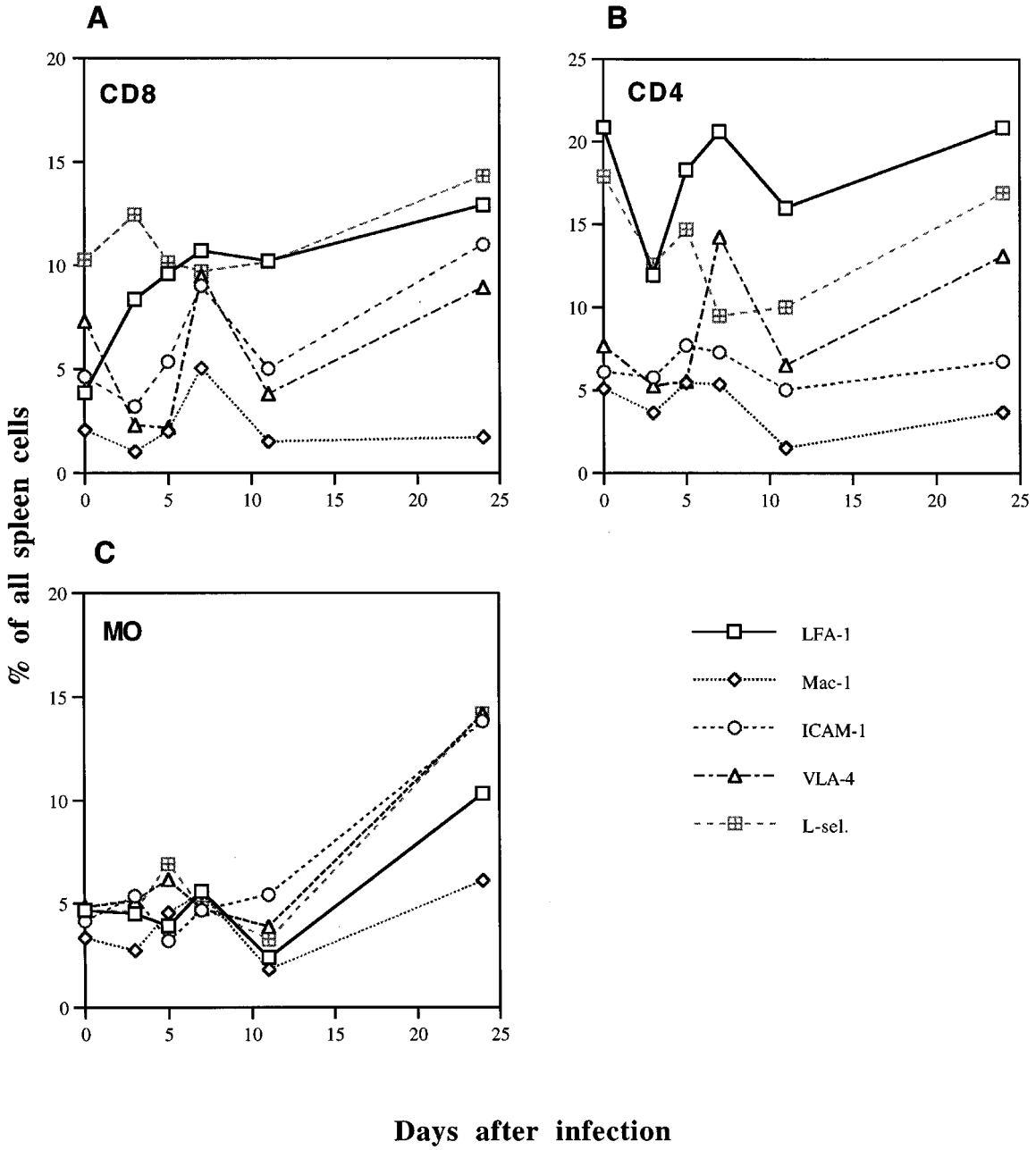

Figure 1 Kinetics of adhesion molecule expression on splenic $CD4^+$ cells, $CD8^+$ cells and macrophages (MO) during SFV-A7 infection. Spleen mononuclear cells were stained with rat mAbs against murine adhesion molecules and FITC-conjugaged anti-rat IgG followed by anti-CD4-PE or anti-CD8-PE or with biotinylated anti-F4/80 and streptavidin-PE. Stained cells were analyzed by flow cytometry. The numbers for each % of all spleen cells represent percentages of double positive cells of all spleen cells. At each timepoint four infected and four uninfected mice were included in two separate experiments. Mean values of uninfected mice are expressed as timepoint 0. The results shown represent one of the two repeated experiments with similar results.

perivascular locations and expression of VCAM-1 on the white matter vessels had increased from low to moderate (Figure 3). From day 3 the number of Mac-1-positive and LFA-1-positive cells increased and expression of ICAM-1 was induced both on vascular endothelial cells and on infiltrating inflammatory cells. The expression of all three leukocyte integrins and their endothelial ligands peaked between days 5–8 after infection (Figure 4b, d and f; Figure 3) and declined thereafter. VCAM-1 was more strictly confined to vascular endothelium than ICAM-1, which was expressed also on the infiltrating inflammatory cells (data not shown). In the most positive areas in the white matter, practically every vessel expressed VCAM-1 (Figure 4b), and strong VCAM-1 expression was also detected on the ependymal cells lining the cerebral ventricles (data not shown). By day 20 expression of Mac-1, ICAM-1, VLA-4 and VCAM-1 had returned to the basal low level or close to it, while foci of strongly LFA-1α-positive cells were still detected in the white matter (Figure 3).

As we have shown also previously (Soilu-Hänninen *et al*, 1994; Erälinna *et al*, 1996), expression of SFV-A7 antigens in the CNS was first detectable at day 3, peaked at day 5, had declined by day 8 and was undetectable at day 20 post infection. Viral antigens were expressed as perivascular foci of infected cells predominantly in the cerebellar and brain stem white matter, but also in cerebral hemispheres and spinal cord (data not shown).

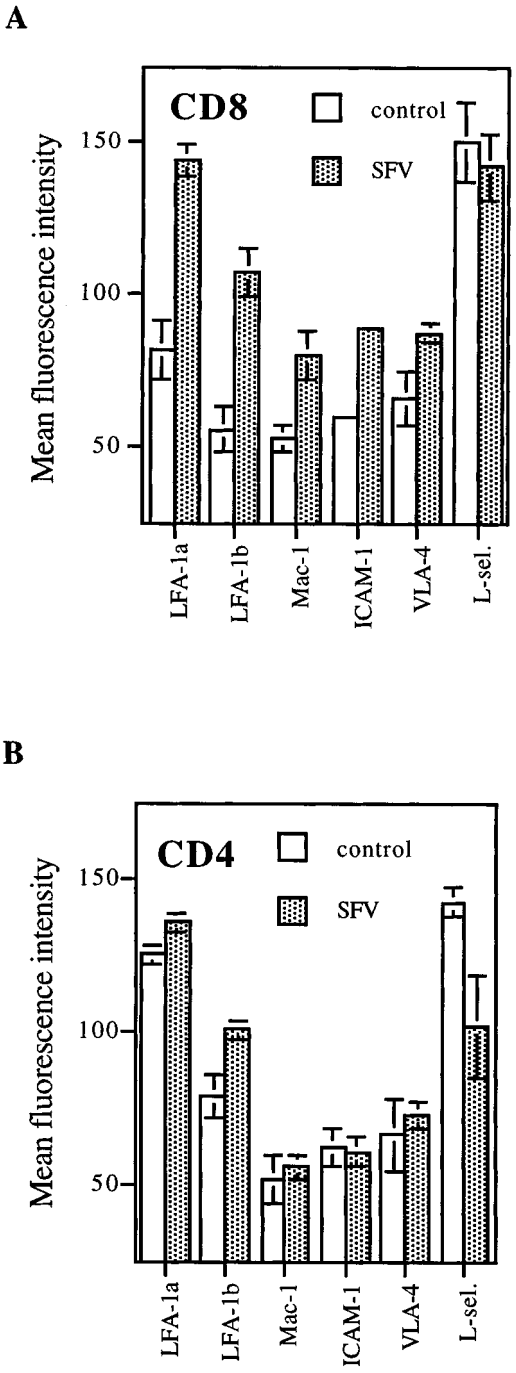

Figure 2 Comparison of the expression of adhesion molecules on the surface of splenic $CD8^+$ cells and $CD4^+$ cells derived from SFV-A7-infected mice at 7 days p.i. and from control mice stained in parallel. Cells were stained with rat mAbs against murine adhesion molecules followed by FITC-conjugated anti-rat IgG and CD8-PE or CD4-PE. Analysis was done by flow cytometry and results represent logarithmic mean fluorescence intensity (MFI) values on green channel of all viable PE-positive. The average and the range of the MFI values obtained in two separate experiments are shown.

In vivo *treatment of SFV-A7-infected mice with mAbs against ICAM-1, VCAM-1 and anti-human CD44 (control)*

To directly demonstrate the contribution of LFA-1/ICAM-1 and VLA-4/VCAM-1 pathways in homing of leukocytes into the CNS, SFV-A7-infected mice were treated with i.p. injections of anti-ICAM-1, anti-VCAM-1 or IgG2a control (anti-human CD44) mAbs between days 1 and 7 after infection. Fourteen mice were included in each treatment arm, and eight mice of each group were analyzed immunohistochemically. Treatment with anti-VCAM-1 mAbs statistically significantly reduced the expression of VLA-4 in the CNS (*P*-value 0.0465 in unpaired Student's *t*-test). There was also a trend towards decreased expressions of LFA-1 in anti-VCAM-1-treated mice and both LFA-1 and VLA-4 in anti-ICAM-1-treated mice, but these changes were not statistically significant (*P*-values greater than 0.05). The results from the immunohistochemical analysis are presented in Figure 5.

Discussion

During SFV-A7 encephalitis, mononuclear cells infiltrate into the CNA and lead to immune-mediated demyelination. An essential step in the formation of the CNS mononuclear infiltrates is binding of circulating leukocytes to the endothelial cells of the

cerebral vessels and subsequent migration through the vessel wall. Complex interactions between adhesion receptors on the surface of the leukocytes and their endothelial ligands take place in the process of binding and transendothelial migration (Springer, 1994). The mechanisms leading to accumulation of mononuclear leukocytes into the CNS during SFV-A7 infection have been undefined. In this paper we studied the expression of adhesion molecules on splenic leukocyte subpopulations and in the CNS during SFV-A7 encephalitis in adult BALB/c mice. The results indicate marked changes in

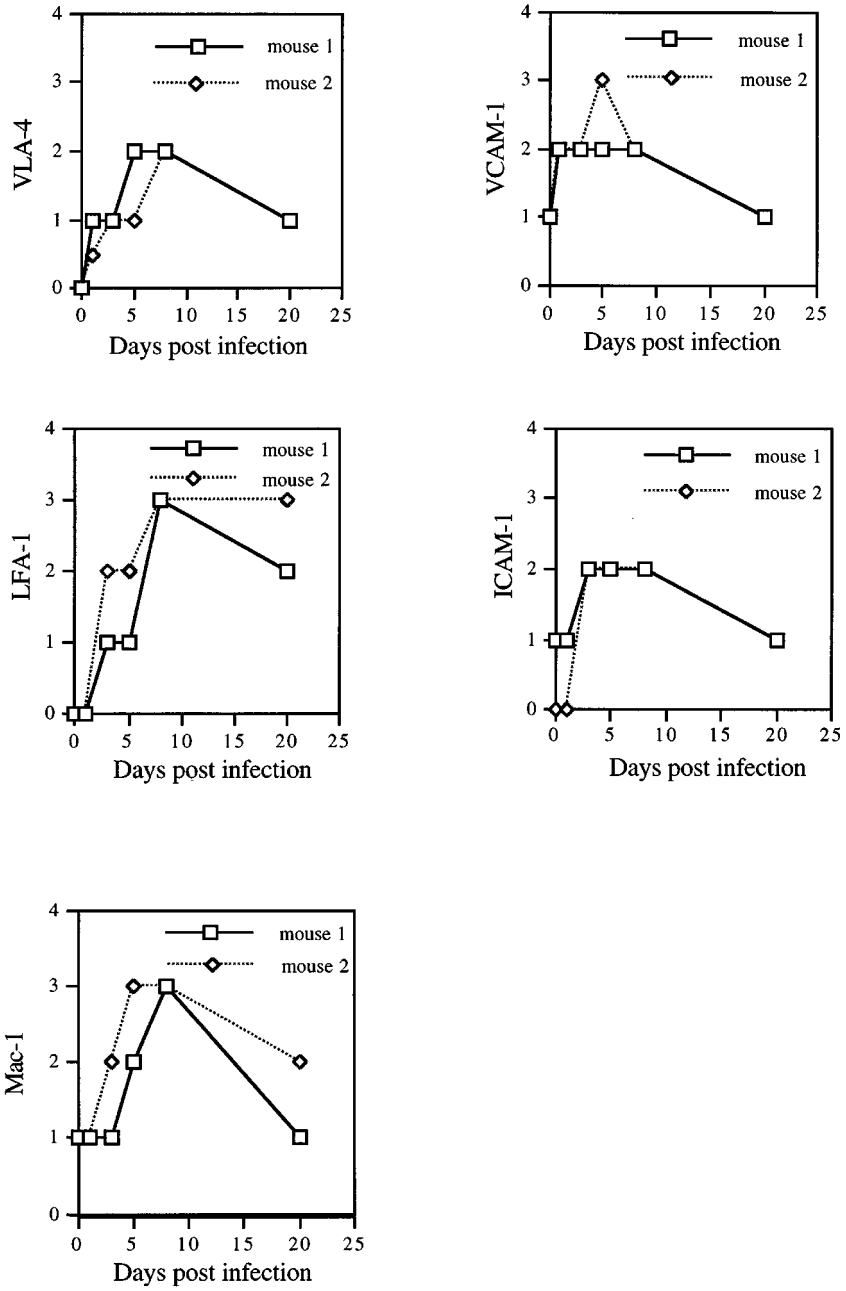

Figure 3 Expression of leukocyte and endothelial cell adhesion molecules in the CNS during SFV-A7 infection. Mice were infected with intraperitoneal injection of 1×10^6 PFU on SFV-A7 in PBS. Frozen sections were prepared from the brain and spinal cord of two infected mice at indicated timepoints and from two mock-infected, age and sex matched control mice. Immunoperoxidase staining was done with mAbs against adhesion molecules and the amount of each antigen was assessed in a blinded fashion on a following semi-quantitative scale: 0, no staining; 1, a few positive cells/weak staining intensity; 2, moderate; 3, many positive cells/strong staining intensity. Two infected mice at days 1, 3, 5, 8 and 20 and two control mice (=day 0) were analyzed, and scores of both mice are shown.

the adhesion molecule profile of splenic $CD8^+$ cells and activation of the CNS vascular endothelium corresponding with the formation and resolution of the inflammatory cell infiltrates.

The infected mice remained clinically asymptomatic. In the spleen, a marked and longlasting increase in the density of LFA-1 on $CD8^+$ cells and increased percentage of LFA-1-expressing $CD8^+$

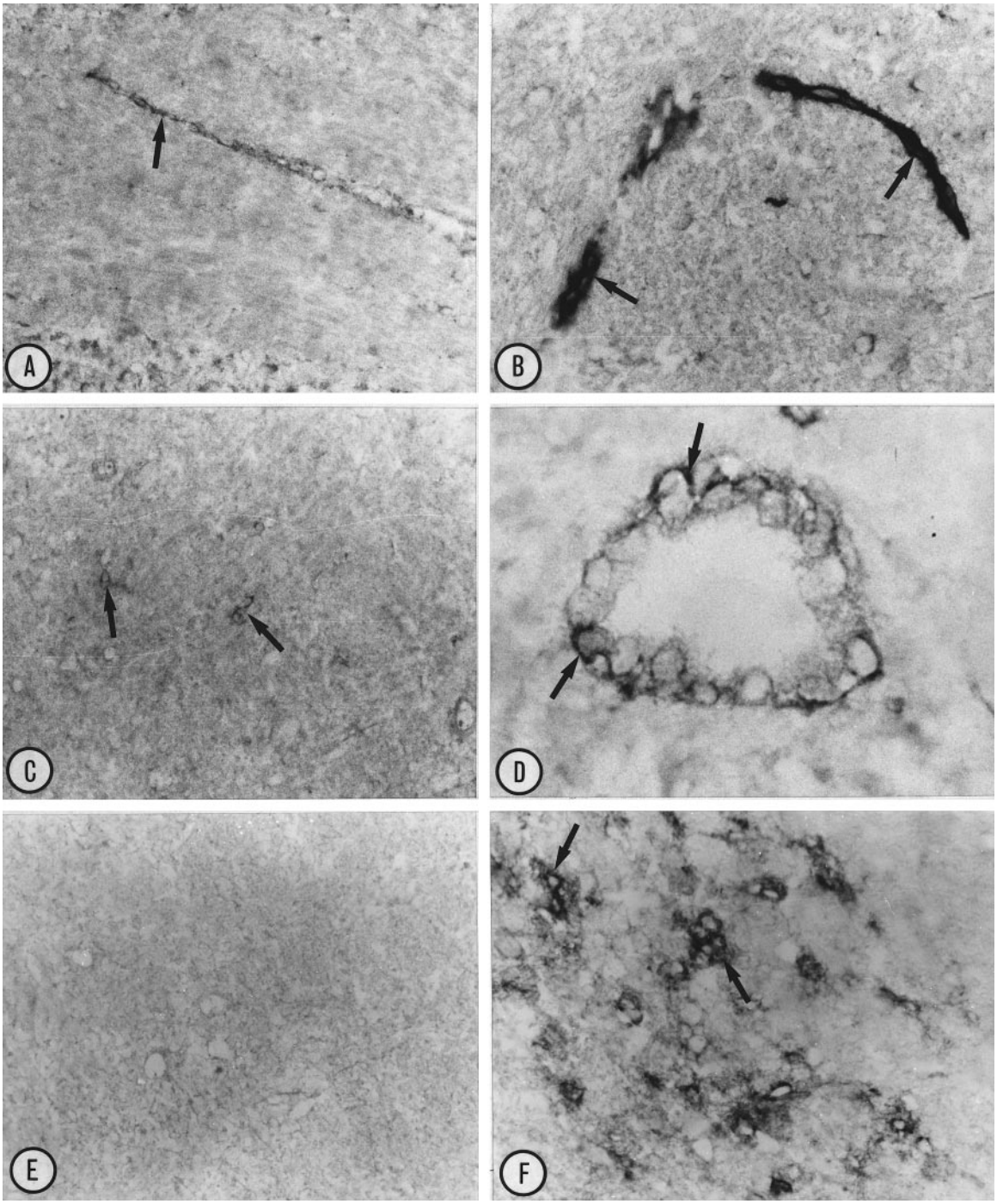

Figure 4 Expression of adhesion molecules in the CNS of SFV-A7 infected and control BALB/c mice. Immunoperoxidase staining was done on frozen sections of brain and spinal cord with rat mAbs against murine adhesion molecules. (**A**) Low basal expression of VCAM-1 on a cerebellar vessel (arrow) of a control mouse. (**B**) Induction of VCAM-1 expression in the cerebellum of an SFV-A7 infected mouse at 5 days p.i. with practically all vessels (arrows) being positively stained. (**C**) Weak or undetectable VLA-4 expression in the CNS of an uninfected mouse. (**D**) Strongly VLA-4-positive cells (arrow) perivascularly in the cerebellum at 8 days p.i. (**E**) Undetectable LFA-1-expression in the CNS of control mice. Area shown is from the cerebellum. (**F**) Numerous strongly LFA-1+ cells (arrows) in the tissue parenchyma in the cerebellum of an SFV-A7 infected mouse at 8 days p.i. (Microscopic magnification: $\times 250$ in A, B, C, E, F; $\times 400$ in d.

cells of all spleen cells was observed. Increased expression of LFA-1 on CD8+ cells has earlier been demonstrated during lymphocytic chorionmeningitis (LCMV) infection in mice (Andersson *et al*, 1995) and infectious mononucleosis in humans (Pallis *et al*, 1993). It may thus represent a phenomenon generally associated with systemic virus infections to augment extravasation of T-cells to the sites of infection. Increased LFA-1 on CD8+ cells may also augment T-cell mediated cytotoxic responses. Supporting this, the adhesion pathway used by virus-specific cytotoxic lymphocytes in adherence to the target cells has been shown to be LFA-1/ICAM-1 but not CD28/B7 (de-Waal-Maletyf *et al*, 1993). Temporary increase in the expression of LFA-1 on CD4+ cells was observed at day seven after infection. Increased LFA-1 on CD4+ cells may be relevant for T-helper responses needed to augment production of virus-specific antibodies (Fischer *et al*, 1986).

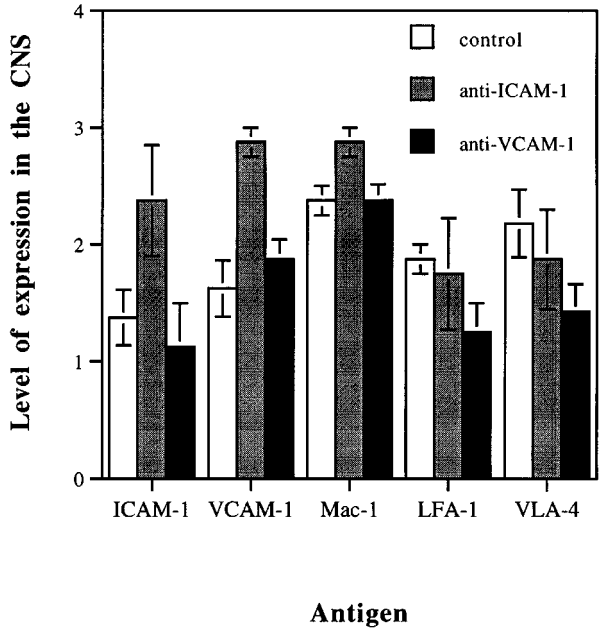

Figure 5 Effect of *in vivo* treatment with mAbs against ICAM-1, VCAM-1 and rat IgG2a control mAb on the expressions of leukocyte and endothelial cell adhesion molecules in the CNS during SFV-A7 infection. Briefly, BALB/c mice were infected i.p. with 1×10^6 PFU/ml of SFV-A7. Subsequently, 100 μg of each mAb was injected i.p. at days 1, 3, 5 and 7 p.i. Each treatment group consisted of 14 mice. Frozen sections were prepared at day 8 p.i. of eight mice in each group and expressions of LFA-1, Mac-1, VLA-4, ICAM-1 and VCAM-1 were determined as described in the Materials. The data are expressed as mean $\pm$ s.e.m. values of eight mice.

Table 1 List of rat mAbs against murine antigens used in this study

mAb/clone	*Antigen recognized*
M17/5.2	LFA-1α/CD11a[a]
M18/2.a	LFA-1β/CD18[a]
M1/70	Mac-1/CD11b[a]
PS/2	VLA-4/CD49d[a]
Mel-14	L-selectin/CD62L[a]
YN/1.7.4	ICAM-1/CD54[a]
Anti-VCAM-1	VCAM-1/CD106[b]
9B5	human CD44[c]
Anti-L3T4-PE	CD4[d]
Anti-Ly-2-PE	CD8[d]
F4/80	160 kD antigen on mouse macrophages[e]

Source of antibodies: [a]American Type Culture Collection (ATCC; Rockville, MD); [b]PharMingen, San Diego, CA; M/K-2.7, ATCC [c]National Public Health Institute, Turku, Finland; [d]Becton Dickinson, San Jose, CA; [e]Boehringer Mannheim GmbH, Germany

At day 7 after infection, also the expressions of VLA-4, Mac-1 and ICAM-1 were increased on CD8+ cells. Increased expression of LFA-1, VLA-4 and ICAM-1 have been shown to be a consequence of activation of murine T cells by antigen or mitogen (Mobley *et al*, 1994). Mac-1 expression seems to be a marker for CD8+ cytotoxic T-cell activation and memory in virus infection (MacFarland *et al*, 1992). The timepoint of increased adhesion molecule expression corresponds to the previously published peak in the production of interferon-γ by spleen cells from SFV-A7 infected mice. This peak was shown to be temporarily associated with cytotoxicity specifically directed against virus-infected targets (Blackman and Morris, 1984). The altered adhesion pattern of CD8+ cells at seven days after infection could indicate appearance of virus-specific cytotoxic T-cells within the CD8+ cell population. Increased expression of VLA-4 and Mac-1 on these cells may direct them into the CNS (Baron *et al*, 1993; Andersson *et al*, 1994; Nielsen *et al*, 1994).

Percentage of splenic CD4+ cells expressing the lymph node homing receptor L-selectin (Mel-14/CD62L) of all spleen cells was decreased during SFV-A7 infection. This may have been caused by downregulation of L-selectin from the surface of a subpopulation of the CD4+ cells. Downregulation of L-selectin from the surface of naive CD4+ T-cells has been associated with their transition from virgin to memory cells (Bradley *et al*, 1992). On CD8+ cells, we did not observe a clear decrease in L-selectin expression, although changes in the expression of the adhesion molecules, associated with cellular activation occurred. However, it has been shown previously, that in contrast to CD4+ memory T cells, the majority of memory CD8+ cells are L-selectin+ and downregulate L-selectin only after restimulation by antigen (Mobley *et al*, 1994). It is possible, that the SFV-A7 specific CD8+ cells in the spleen are L-selectin+ and downregulate L-selectin after contact with virus antigen in the CNS.

We have earlier shown, that expression of ICAM-1 is increased in the CNS during SFV-A7 infection (Soilu-Hänninen *et al*, 1994). We now demonstrate that also VCAM-1 is induced on the endothelial

cells in cerebral blood vessels and on the ependymal cells lining the cerebral ventricles during SFV-A7 infection. Treatment of SFV-A7-infected mice with anti-ICAM-1 or anti-VCAM-1 slightly reduces the amount of inflammatory cells expressing LFA-1 and VLA-4 in the CNS. The differences were, however, not statistically significant, except for VLA-4-expression after anti-VCAM-7-treatment. This could have been due to the limited numbers of mice that were analyzed in each group.

SFV-A7 infection facilitates the development of experimental allergic encephalomyelitis (EAE) in BALB/c mice (Wu *et al*, 1988). We have previously shown that increased entry of inflammatory cells into the CNS is involved in this enhancement of EAE (Erälinna *et al*, 1994). Moreover, the viral facilitation of EAE can be substantially alleviated by *in vivo* treatment with anti VLA-4 and anti-ICAM-1 mAbs (Soilu-Hänninen *et al*, 1996). Taken together with our current results this suggests that both LFA-1/ICAM-1 and VLA-4/VCAM-1-pathways may be functionally involved in leukocyte entry into the CNS during SFV-A7 infection.

Induction of VCAM-1 on CNS blood vessels has earlier been demonstrated during LCMV meningitis (Marker *et al*, 1995) and during Sindbis-virus encephalitis (Irani and Griffin, 1996) in mice and during SIV encephalitis in macaques (Sasseville *et al*, 1992). Later it was demonstrated in the SIV model using a tissue adhesion assay that human monocytic cells only bound to VCAM-1-positive vessels and that binding could be inhibited by preincubation with antibodies to VLA-4 (Sasseville *et al*, 1994). This suggest that the VLA-4/VCAM-1-interaction is the primary adhesion pathway for leukocyte entry into the CNA during SIV encephalitis similarly as has earlier been demonstrated for autoimmune inflammation of the CNS in experimental allergic encephalomyelitis (Yednock, 1992; Baron, 1993). In contrast with these findings, Irani and Griffin (1996) could only inhibit lymphocyte entry into the CNS during Sindbis-virus infection by anti-LFA-1 antibodies, while anti-VLA-4 antibodies were ineffective.

Concomitantly with the upregulation of ICAM-1 and VCAM-1 in the cerebral vessels we observed infiltration of inflammatory cells expressing their integrin ligands LFA-1 or Mac-1 and VLA-4, respectively. A peak in the expression of all the adhesion molecules studied in the CNS occurred between days 5 and 8 post infection. This corresponds to the increased adhesion molecule expression on splenic T cells and detection of viral antigens in the CNS. Interestingly, activation of the CNS endothelium was most prominent in the areas where the viral antigens were predominantly expressed, namely the cerebellar and brain stem white matter. It is possible that the altered adhesion molecule pattern of splenic T-cells directs them into the CNS. Upon contact with SFV-A7 antigens in the brain the virus-specific T-cells may get further activated and secrete cytokines which upregulate adhesion molecules on adjacent blood vessels. Activation of the vascular endothelium in the areas where specific virus antigen recognition takes place may direct circulating monocytes into the infected areas of the CNS.

In summary, following intraperitoneal infection of immunocompetent, adult mice with SFV-A7 virus, the adhesion molecule profile of splenic CDS^+ cells and, to a lesser extent, $CD4^+$ cells is changed. This is temporarily associated with infiltration of inflammatory cells into the CNS and induction of adhesion molecule expression in the brain vessels. We hypothesize that the virus-specific T-cells first enter into the CNS due to changes in their adhesion molecule expression. After contact with their specific antigen there they secrete cytokines and further prime the CNS endothelium for recruitment of monocytes/macrophages into the CNS. After resolution of most of the inflammatory changes, brightly $LFA\text{-}1^+$ cells are still present in the white matter. They may represent the virus-specific cytotoxic T-cells which are thought to be responsible for the demyelination during SFV-A7 infection.

Materials and methods

Mice

BALB/c mice were raised at the Central Animal Laboratory of the University of Turku from breeder stocks obtained from Harlan Sprague-Dawley, Inc., USA. Female mice at age 6–8 weeks were used for virus infection. All animal experiments were approved by the ethics committee of the local state authorities.

Virus

An avirulent mutant of Semliki Forest virus (SFV-A7) was obtained from Dr HE Webb (Neurology Unit, Department of Neurology, Rayne Institute, St. Thomas' Hospital, London, UK), plaque purified three times and amplified in a BALB/c mouse brain cell line (MBA-1) previously established in our laboratory. Virus was titrated in another BALB/c brain cell line (MBA-13) using a standard plaque assay. The virus was stored in 1 ml aliquots at $-70^{\circ}C$ until used at 1×10^6 plaque forming units (PFU)/ mouse injected intraperitoneally (i.p.) in 100 μl of sterile phosphate-buffered saline (PBS).

Cell preparations

Spleens were removed from mice killed by CO_2 anaesthetization. Single cell suspensions were prepared by pressing the spleens through a fine steel mesh. The red blood cells were lysed by 0.83% NH_3CL treatment.

Antibodies
Monoclonal antibodies (mAbs) against adhesion molecules and murine leukocyte subset markers used in this paper are listed in Table 1. Rabbit polyclonal antibodies against SFV-A7 were raised in our laboratory by immunization with inactivated SFV-A7 virus. For double immunofluorescence staining the murine macrophage marker, mAb F4/80, was biotinylated with biotin-N-hydroxysuccinimide (Zymed, San Francisco, CA, USA) as described earlier (Bayer and Wilcheck, 1978). Streptavidin-conjugated phycoerythrin (SA-PE) was from Becton Dickinson, San Jose, CA, USA. FITC-conjugated affinity-purified goat anti-rat IgG was from Cappel, Durham, NC, USA. Vectastain anti-rat and anti-rabbit antibody staining kits used in immunohistochemistry were from Vector Laboratories, Inc., Burlingame, CA, USA.

Production of mAbs for in vivo *injections*
For production of mAbs for *in vivo* injections, the anti-VCAM-1 (M/K-2.7), anti-ICAM-1(YN/1.7.4) and anti-human CD44 (9B5) mAb producing hybridomas were grown in serum free and protein free growth medium (Catalog No. S-2772, Sigma, St Louis, MO, USA). Protein was precipitated from cell-free supernatants with saturated ammonium-sulphate (340 g/l of $(NH4)_2SO_4$ at +4°C) and dialyzed against PBS. Purity of the mAbs was verified by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS–PAGE) and all aliquots to be used were essentially free from contaminating proteins. Protein concentrations were determined by Ultrospec K 4053 spectrophotometer (LKB Biokrom, Cambridge, England) based on optical density values at 280 nm. MAbs were adjusted to a final concentration of 1 mg/ml in PBS and sterilized by filtration (0.2 μm filter from Millipore, Bedford, MA). Total of 0.1 mg (100 μl) of mAb was injected intraperitoneally into mice every other day between days 1 and 7 p.i.

Flow cytometry
For analysis of expression of adhesion molecules on spleen $CD8^+$ and $CD4^+$ cells and macrophages, freshly isolated spleen cells were washed with PBS containing 2% fetal calf serum (FCS) and 0.1% sodium azide (wash medium). Aliquots of 1×10^6 cells were then incubated with saturating concentrations of purified, concentrated mAbs against LFA-1α, Mac-1, LFA-1β, ICAM-1, VLA-4 and L-selectin diluted in wash medium. Cells were then washed twice and incubated with FITC-conjugated goat anti rat-IgG containing 5% normal mouse serum. The cells were washed again and incubated further with PE-conjugated anti-CD4 or anti-CD8 mAbs or with biotinylated F4/80 mAb. To inhibit background staining caused by binding of the FITC-conjugated anti-rat IgG to the PE-conjugated rat anti-CD4 and anti-CD8 mAbs, 5% normal rat serum was added to the anti-CD4 and anti-CD8 mAbs. After the incubation the anti-CD4 and anti-CD8-stained cells were washed and fixed with PBS containing 3% paraformaldehyde. Cells stained with the biotinylated anti-mouse macrophage marker were further incubated with SA-PE before final washes and fixation.

The stained cells were then analyzed using fluorescence activated cell sorter (FACSscan, Becton Dickinson, Mountain View, CA). A total of 10 000 viable cells were acquired from each sample using the forward scatter and side scatter parameters to exclude dead cells and debris. Each cell was scored for fluorescence intensity on red and green channels to determine the expression of the leukocyte subset marker and the adhesion molecule marker, respectively. Analysis was performed with Lysys 1.6 computer program. The results are presented as percentages of double positive cells of all spleen cells or as mean fluorescence intensity values of all PE-positive cells on the green channel. Splenocytes from uninfected, age and sex-matched mice were stained in parallel as controls. Four infected and four control mice were studied at each timepoint in two separate experiments.

Immunohistochemistry
For preparation of frozen sections, two infected mice at days 1, 3, 5, 8 and 20 after infection and two control mice were extensively perfused with PBS under anesthesia. The brains and the spinal cords were then removed and rapidly frozen in Tissue Tek (Miles Inc., Naperville, Ill.) by immersion in liquid nitrogen. The blocks were stored at −70°C until use. Frozen sections were cut on a cryotome at a 6 μm thickness, mounted on organosiliconized slides (Maples, 1985), air dried, fixed in cold acetone for 10 min and preserved at −20°C until use. For immunoperoxidase staining rat mAbs against murine LFA-1, Mac-1, VLA-4, ICAM-1 and VCAM-1 or rabbit polyclonal anti-SFV, were allowed to react on the sections overnight at +4°C. Frozen sections of mice that had been treated with anti-ICAM-1, anti-VCAM-1 or control mAbs were first incubated for 30 min with nonconjugated goat anti-rat IgG to block cross-reaction with the mAbs injected *in vivo*. For virus antigen detection, endogenous peroxidase was blocked by 30 min incubation with 0.3% H_2O_2 in methanol prior incubation with 1:2000 dilution of rabbit anti-SFV-antibody. Binding of primary mAbs was visualized by ABC-technique with Vectastain anti-rat or anti-rabbit antibody staining kits. Color reaction was developed with DAB. The sections were counterstained with haematoxylin. The number of positively stained cells and their staining intensity was evaluated in a blinded fashion and scored on a following semiquantita-

tive scale: 0, no reactive cells; 1, a few positive cells/weak staining; 2, moderate; 3, many positive cells on every section/strong staining intensity. Approximately ten sections/mouse were studied. Statistical significance in group comparisons was determined by Student's *t*-test.

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References

Amor S, Scallan MF, Morris MM, Dyson H, Fazakerley JK (1996). Role of immune responses in protection and pathogenesis during Semliki Forest virus encephalitis. *Gen Virol* **77:** 281–291.

Andersson EC, Christensen JP, Marker O, Thomsen AR (1994). Changes in cell adhesion molecule expression on T cells associated with systemic virus infection. *J Immunol* **152:** 1237–1245.

Andersson EC, Christensen JP, Scheynius A, Marker O, Thomsen AR (1995). Lymphocytic choriomeningitis virus infection is associated with long-standing perturbation of LFA-1 expression on CD8+ T cells. *Scand J Immunol* **42:** 110–118.

Baron JL, Madri JA, Ruddle NH, Hashim G, Janeway CA (1993). Surface expression of alpha4 integrin by CD4 T-cells is required for their entry into brain parenchyma. *J Exp Med* **177:** 57–68.

Barten DM, Ruddle NH (1994). Vascular cell adhesion molecule-1 modulation by tumor necrosis factor in experimental allergic encephalomyelitis. *J Neuroimmunol* **51:** 123–133.

Bayer E, Wilcheck M (1978) The avidin biotin complex as a tool in molecular biology. *Trends Biochem Sci* **3:** N257.

Berger M (1980). Humoral and cell mediated immune mechanisms in the production of pathology in the avirulent Semliki Forest Virus. *Inf Immun* **30:** 244–253.

Blackman MJ, Morris AG (1984). Gamma interferon production and cytotoxicity of spleen cells from mice infected with Semliki Forest virus. *J Gen Virol* **65:** 955–961.

Bradley LM, Atkins GG, Swain SL (1992). Long-term CD4+ memory T cells from the spleen lack MEL-14, the lymph node homing receptor. *J Immunol* **148:** 324–331.

Butcher EC (1991). Leukocyte-endothelial cell recognition: three (or more) steps to specificity and diversity. *Cell*, **67,** 1033–1036.

De-Waal-Maletyf R, Verma S, Bejarano MT, Ranes-Goldberg M, Hill M, Spits H (1993). CD2/LFA-3 or LFA-1/ICAM-1 but not CD28/B7 interactions can augment cytotoxicity by virus-specific CD8+ cytotoxic T lymphocytes. *Eur J Immunol* **23:** 418–424.

Erälinna JP, Soilu-Hänninen M, Röyttä M, Ilonen J, Mäkelä M, Salmi AA, Salonen R (1994). Facilitation of experimental allergic cncephalomyelitis by irradiation and virus infection: role of inflammatory cells. *J Neuroimmunol* **55:** 81–90.

Erälinna JP, Soilu-Hänninen M, Röyttä M, Hukkanen V, Salmi AA, Salonen R (1996). Blood-brain barrier breakdown and increased expression of intercellular adhesion molecule-1 (ICAM-1) after Semliki Forest virus (A7) infection facilitate the development of experimental allergic encephalomyelitis. *J Neuroimmunol* **66:** 103–114.

Fazakerley JK, Webb HE (1987). Semliki Forest virus-induced immune-mediated demyelination: adoptive transfer studies and viral persistence in nude mice. *J Gen Virol* **68:** 377–385.

Fisher A, Durandy A, Sterkers G, Griscelli C (1986). Role of the LFA-1 molecule in cellular interactions required for antibody production in humans. *J Immunol* **136:** 3198–3023.

Greenwood J, Wang Y, Calder VL (1995). Lymphocyte adhesion and transendothelial migration in the central nervous system: The role of LFA-1, ICAM-1, VLA-4 and VCAM-1. *Immunology* **86:** 408–415.

Irani DN, Griffin DE (1996). Regulation of lymphocyte homing into the brain during viral encephalitis at various stages of infection. *J Immunol* **156:** 3850–3857.

MacFarland HI, Nahill SR, Maciaaszek JW, Welsh RM (1992). CD11b (Mac-1): a marker for CD8+ cytotoxic T cell activation and memory in virus infection. *J Immunol* **149:** 1326–1333.

Maples JA (1985). A method for the noncovalent attachement of cells to glass slides for use in immunohistochemical assays. *Am J Clin Pathol* **83:** 356–363.

Marker O, Scheyniues A, Christensen JP, Thomsen AR (1995) Virus-activated T cells regulate expression of adhesion molecules on endothelial cells in sites of infection. *J Neuroimmunol* **62:** 35–42.

Mobley JL, Rigby SM, Dailey MO (1994). Regulation of adhesion molecule expression by CD8 T cells in vivo. *J Immunol* **153:** 5443–5452.

Nielsen HV, Christensen JP, Andersson EC, Marker O, Thomsen AR (1994). Expression of type 3 complement receptor on activated CD8+ T-cells facilitates homing to inflammatory sites. *J Immunol* **153:** 2021–2028.

Osborn L (1990). Leukocyte adhesion to endothelium in inflammation. *Cell* **62:** 3–6.

Pallis M, Robins A, Powell R. (1993). Quantitative analysis of lymphocyte CD11a using standardized flow cytometry. *Scand J Immunol* **38:** 559–564.

Pober J, Cotran R (1990). The role of endothelial cells in inflammation. *Transplantation* **50:** 537–544.

Sasseville V, Newman W, Lackner A, Smith M, Lausen N, Beall D, Ringler D (1992). Elevated vascular cell adhesion molecule-1 in AIDS encephalitis induced by Simian immunodeficiency virus. *Am J Pathol* **141:** 1021–1030.

Sasseville VG, Newman W, Brodie SJ, Hesterberg P, Payley D, Ringler DJ (1994). Monocyte adhesion to endothelium in Simian Immunodeficiency Virus-induced AIDS encephalitis is mediated by vascular cell adhesion molecule-1/$\alpha 4\beta 1$ integrin interactions. *Am J Pathol* **144:** 27–40.

Sobel R, Mitchell M, Fondren G (1990). Intercellular adhesion molecule-1 (ICAM-1) in cellular immune reactions in the human central nervous system. *Am J Pathol* **136:** 1309–1316.

Soilu-Hänninen M, Erälinna JP, Hukkanen V, Röyttä M, Salmi AA, Salonen R (1994). Semliki Forest virus infects mouse brain endothelial cells and causes blood-brain barrier damage. *J Virol* **68:** 6291–6298.

Soilu-Hänninen M, Röyttä M, Salmi AA, Salonen R (1997). Therapy with antibody against leukocyte integrin VLA-4 is effective and safe in virus-facilitated experimental allergic encephalomyelitis. *J Neuroimmunol* **72:** 95–105.

Springer TA (1994). Traffic signals for lymphocytic recirculation and leukocyte emigration: the multistep paradigm. *Cell* **76:** 301–314.

Subak-Sharpe I, Dyson H, Fazakerley J (1993) In vivo depletion of CD8+ T cells prevents lesions of demyelination in Semliki Forest virus infection. *J Virol* **67:** 7629–7633.

Suckling AJ, Pathak S, Jagelman S, Webb HE (1978). Virus-associated demyelination. A model using avirulent Semliki Forest virus infection of mice. *J Neurol Sci* **39:** 147–154.

Willenborg DO, Simmons RD, Tamatani T, Miyasaka M (1993). ICAM-1-dependent pathway is not critically involved in the inflammatory process of autoimmune encephalomyelitis or in cytokine-induced inflammation of the central nervous system. *J Neuroimmunol* **45:** 147–154.

Wu L-X, Mäkelä MJ, Röyttä M, Salmi A (1988). Effect of viral infection on experimental allergic encephalomyelitis in mice. *J Neuroimmunol* **18:** 139–153.

Yednock T, Cannon C, Fritz LC, Sanchez-Madrid F, Steiman L, Karin N (1992). Prevention of experimental autoimmune encephalomyelitis by antibodies against $\alpha 4\beta 1$ integrin. *Nature (Lond.)* **356:** 63–66.