Supplemental Results

ICAM-1 clustering is critical for downstream signaling

ICAM-1 clustering was induced in GPNT cells the addition of anti-ICAM-1 antibody followed by crosslinking with a secondary goat anti-mouse antibody (Supplemental Figure 1 A). Cluster formation was accompanied by signals inducing actin-rearrangement. Pre-treatment of GPNT with the cholesterol-disrupting drug methyl-β-cyclodextrin, which interferes with membrane domains, resulted in disassembly of ICAM-1 clusters and loss of actin re-arrangements. These data indicate the importance of ICAM-1 clustering for the induction of downstream signaling.

ICAM-1-induced calcium transients in CHO cells

Calcium studies were performed in CHO cells stably expressing human ICAM-1. Addition of anti-human ICAM-1 antibody (clone 15.2) led to oscillating Ca^{2+} release (Supplemental Figure 1 B). Peaks oscillated with an average frequency of 144 +/- 10 s (71 cells, n = 3) and their amplitudes were in the same order of magnitude as when ionomycin was added. Significantly, antibody cross-linking did not induce any further activation of Ca^{2+} release (data not shown). Oscillating calcium transients were specific to ICAM-1 ligation, since it did not occur in the absence of ICAM-1 (in wt CHO cells) or when an isotype control Ab were used.

ROS accumulation GPNT cells

T lymphocytes were allowed to adhere to confluent GPNT cells. Subsequently, ROS were measured using the fluorescent probe CM-H_2DCFDA. Compared to control, significant increase in endothelial ROS production was observed following addition of T cells (Supplemental Figure 2 A). The production of oxygen species relies on different intracellular enzyme complexes such as NADPH Oxidase (NOX), Xanthine Oxidase (XO) and Nitric Oxide

Synthase (NOS). To establish which enzymes played a role in T cells-mediated ROS production, we used specific inhibitors. When GPNT cells were pre-treated with the NOS inhibitor L-NAME prior to the addition of T cells, a total reduction in the production of ROS was observed (Supplemental Figure 2 A). In contrast, neither allopurinol (a XO inhibitor) nor apocynin (a NOX inhibitor) affected ROS production. Since lymphocytes interact with EC via a number of receptors and adhesion molecules, we further analyzed whether ROS production was ICAM-1 mediated. For this, EC lines derived from ICAM-1-/- (bEndI1.1) or wild-type (bEnd.5) mice were used (Supplemental Figure 2 B). A significant increase in the production of oxygen species was observed in wild-type cells 30 min after T cell addition. In contrast, ICAM-1 null EC showed much lower levels of ROS induction suggesting that ICAM-1 was the main mediator of lymphocyte-induced ROS production. Indeed, antibody-mediated ICAM-1 stimulation was sufficient to generate an endothelial ROS response which was similar as when T cells were added (Supplemental Figure 2 C). Significantly, as seen when calcium was measured, subsequent antibody cross-linking using anti-mouse Ig did not further increase ROS levels (data not shown). Moreover, an induction in ROS production was not detected when cells were exposed to isotype control antibodies.

The sensitivity of ROS induction to L-NAME suggested that reactive nitrogen species were predominantly produced. To confirm this, endothelial cells were loaded with DAF-2DA, a dye that fluoresces upon binding to oxidized species of NO. Subsequently, EC were stimulated with 1A29 or insulin, which leads to release of NO in EC (Montagnani *et al.*, 2001). Both insulin and ICAM-1 ligation enhanced the rate at which DAF-2DA fluorescence developed (Supplemental Figure 2 D).

Supplemental Material and Methods

Calcium imaging

Wild-type Chinese hamster ovary (CHO) cells and stable transfectants expressing human ICAM-1 (a kind gift by Prof. Jeremy Pearson, King's College London, UK) were loaded with 5 μM Fluo-4-AM (Molecular Probes) in HBSS for 30 minutes prior to analysis on a Leica TCS SP2 AOBS confocal microscope using a 40x oil immersion lens. Fluo-4 was excited using the 488 nm line of an argon laser and the emitted light was collected between 510 nm-600 nm. For time-resolved analyses, images were collected approximately every 1.5 s. Fluorescence traces (arbitrary units) from individual cells were recorded from three independent experiments and analyzed using Leica confocal software.

Measurement of Reactive Oxygen Species production

Cells were grown in 96-well plates and, at confluence, cultured in serum-free medium overnight. Medium from each well was removed and replaced with pre-warmed phenol-red free HBSS containing the fluorescent dye 5-6-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate acetyl ester (CM-H_2DCFDA, Molecular Probes, 5 μM, 100 μl/well) for 60 minutes. After incubation, cells were washed twice with pre-warmed HBSS before stimulation. The fluorescence of each well was measured in a fluorescence plate reader (excitation 490 nm, emission 520 nm). Results were obtained by subtracting the reading of controls from the correspondent reading for every time point following stimulation.

Lymphocyte adhesion in vitro

Lymphocyte adhesion assays on immortalized Lewis rat brain endothelial cells (GPNT) were conducted as previously described (Turowski *et al.*, 2008).

Supplemental Figure Legends

Supplemental Figure S1. ICAM-1-induced calcium release in CHO cells. (A) ICAM-1 clustering and induced cytoskeletal rearrangement. GPNT cells were either left untreated (*a* to *d*), or pretreated with 10 mM Methyl-β-cyclodextrin for 30 min (*e* to h), Where indicated, cells were subjected to ICAM-1 cross-linking (*b, d, f,* and *h*) for 10 min. Cells were fixed and stained for ICAM-1 and actin. Results are representative of three independent experiments. (B) Wild-type (control; bottom right hand trace) or CHO cells expressing human ICAM-1 were loaded with Fluo-4AM for 30 min and Ca^{2+} fluctuations in individual cells monitored by confocal microscopy (see Material and Methods). Where indicated cells were stimulated using 10 μg/ml ICAM-1 antibody or IgG1 isotype control antibody. At the end of each experiment cells were further stimulated with 2 μM ionomycin. Shown is the time-resolved Fluo-4 specific increase in fluorescence (arbitrary unit F = 10) of one representative cell from each of three independent experiments.

Supplemental Figure S2. ICAM-1 engagement induces production of reactive oxygen species and nitric oxide in endothelial cells. (A) GPNT cells were pre-treated with the XO inhibitor allopurinol (Allo, 100 μM), the NOX inhibitor apocynin (Apo, 600 μM) or the NOS inhibitor L-NAME (600 μM) for 1 h. EC were washed, T cells added and ROS production determined after 30 min. (B) As in panel A, except that wild-type (bEnd.5) or ICAM-1-deficient (bEnd.I1.1) mouse brain endothelioma cells were used. (C) As in panel A, except that ROS production was measured following the addition of 10 μg/ml anti-ICAM-1 antibody 1A29 or isotype control IgG1 (anti-MHC-I). (D) GPNT cells were stimulated either by adding anti-ICAM-1 mAb 1A29 (10 μg/ml) or insulin (Ins, 1 μM) and cellular NO content measured using DAF-2DA. Increase in fluorescence was analysed by linear regression followed by

statistical analysis using ANCOVA. The response to 1A29 or insulin was very significant ($p<0.001$).

Supplemental Figure S3. Effects of NOX and XO inhibitors on lymphocyte migration. GPNT cells were left untreated (Control) or treated with the XO inhibitor Allopurinol (100 μM) or the NOX inhibitor Apocynin (600 μM) prior to migration. All values are mean values ± SEM derived from three to five independent experiments. *, $P < 0.05$; **, $0.001<P< 0.01$, ***, $P \leq 0.001$.

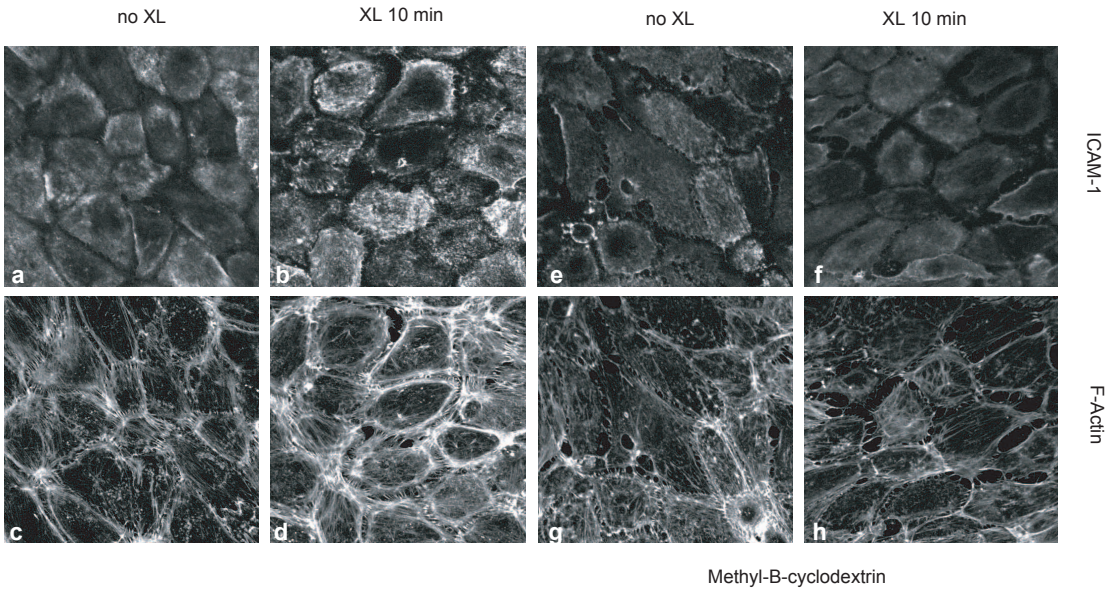
Supplemental Figure S4. Lymphocyte migration in eNOS$^{-/-}$ derived brain endothelium. (A) Migration assay performed as described in Figure 6, except that primary brain EC from wt or eNOS$^{-/-}$ mice were used. (B) Migration assay across EC from eNOS$^{-/-}$ mice as in panel A. Where indicated EC were pretreated using L-NAME (600 μM, 1h).

Supplemental Figure S5. Lymphocyte adhesion to GPNT monolayers. (A-D) GPNT cells were pre-treated as in Figure 6 prior to adhesion experiments. All values are mean values ± SEM derived from three to five independent experiments. *, $P < 0.05$; **, $0.001<P< 0.01$, ***, $P \leq 0.001$.

Supplemental Movies

Role of the AMPK/eNOS signaling pathway during transendothelial lymphocyte migration. GPNT cells were grown to confluence in 96-well tissue culture plates and either left untreated (Control) or treated with L-NAME (600 μM, 1 h) or compound C (20 μM, 30 min). Cells were then extensively washed and incubated with antigen-specific T cells (2 x 10^5/mL). T cells were allowed to adhere and migrate for 30 min, at which point individual wells were subjected to time-lapsed video microscopy over a 5 min period (31 frames). Shown is a representative video (10 frames/s) for each condition. Arrows indicate positions of cells that are phase dark and localized under the EC monolayer ("migrated"). The quantitative data shown in Figure 2, 4, 5 and 7 have been derived from such videos. Cells were counted from 6 wells per condition and every experiment was performed at least 3 times.

A



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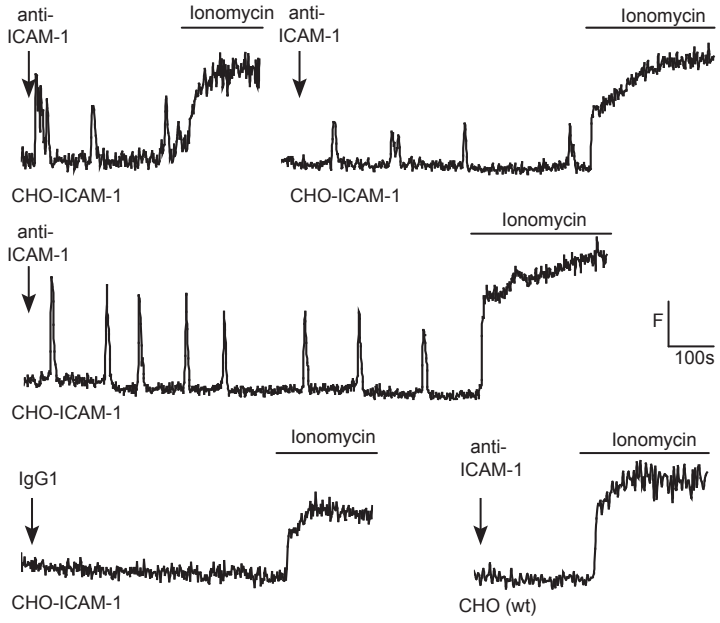
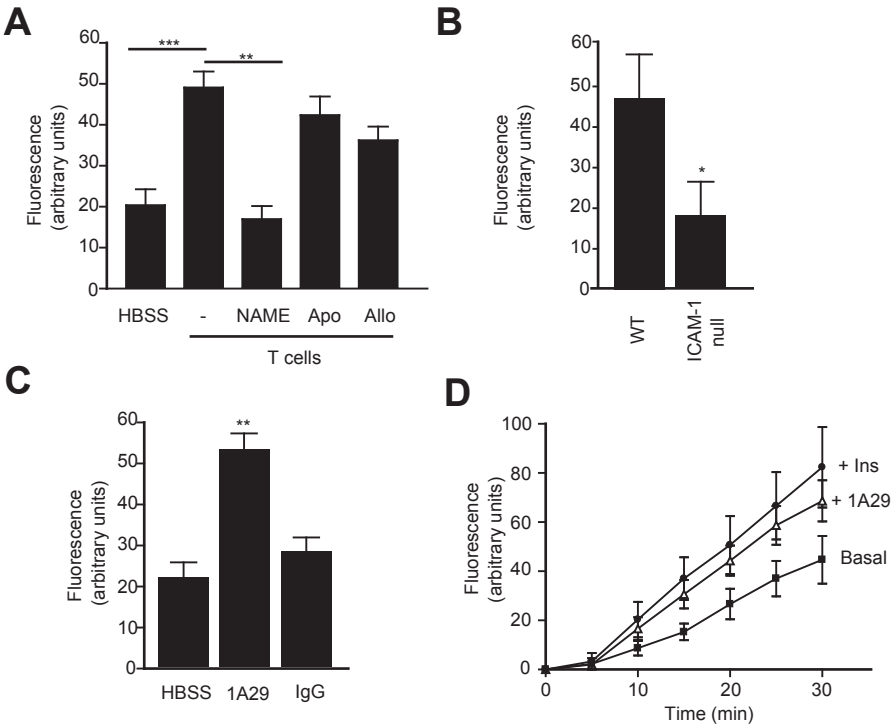
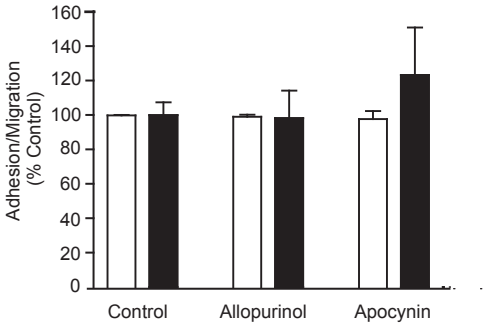


Martinelli et al., Figure S1



Martinelli et al., Figure S2



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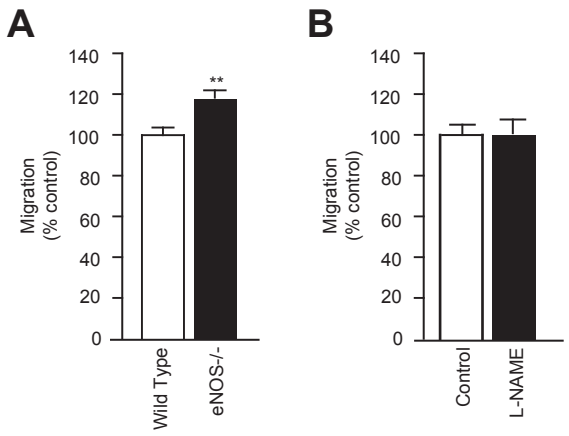

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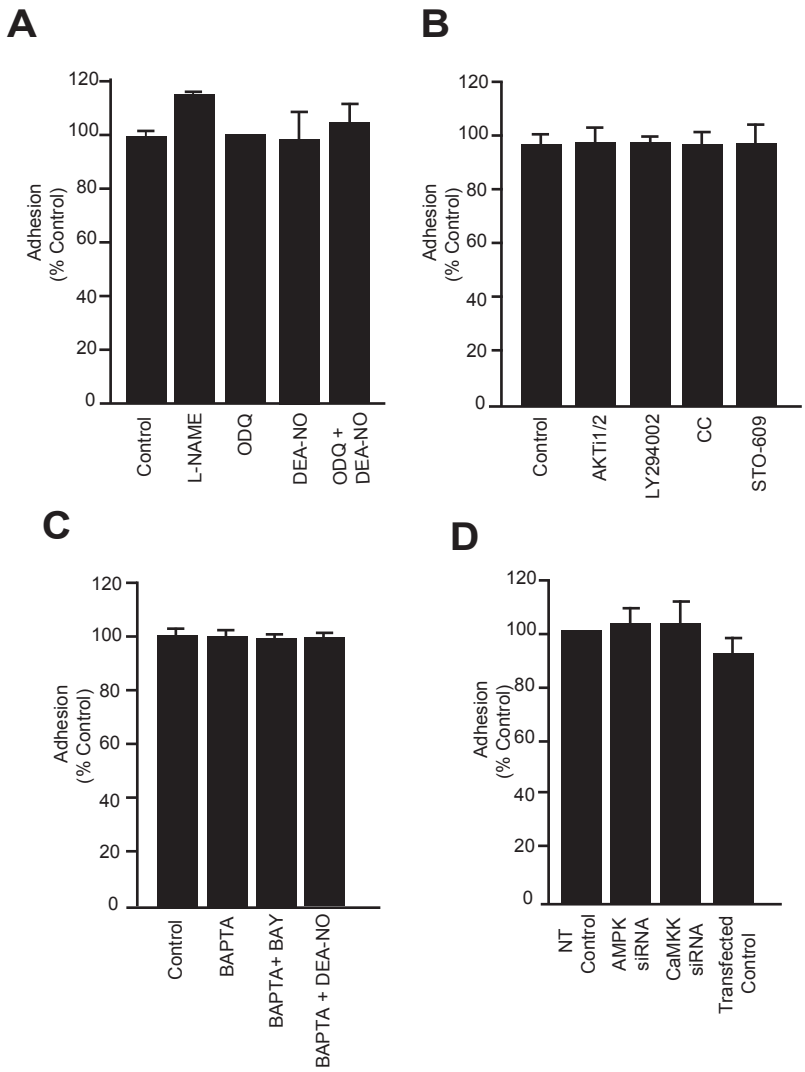

Martinelli et al., Figure S5