ONLINE SUPPLEMENT

α4 Integrin is a Regulator of Leukocyte Recruitment Following Experimental Intracerebral Hemorrhage

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Supplemental Methods

Mice

C57BL/6J wild type mice were purchased from the Jackson Laboratory and then bred in-house. Male mice were used for experiments 8-12 weeks after birth. Forty-five total mice were used in these experiments (8 mice for experiments shown in Figure 1 and 37 mice for experiments shown in Figure 2). Mice were randomized to treatment groups (ICH/sham; control/anti-α4 integrin) by coin-flip.

ICH Model

Mice were maintained under 2-5% isoflurane inhalation anesthesia at 37 ± 1.0°C, as 25 μl of autologous blood were injected 0.5-1.0 μl per minute. The blood was injected in two fractions, pausing for 5 minutes to allow injected blood to clot, 2.5 mm right and 3 mm below bregma at a 5° angle toward the midline. This striatial ICH causes a moderate left forelimb deficit. Brains were evaluated for surgical success at the time of sacrifice and included only if the majority of blood was located in the striatum and minimal blood pooled on the surface of the brain. Shams received the same treatment, including needle insertion, but not blood injection. The investigator responsible for surgeries and assessment of surgical success on antibody-treated mice was blinded to the treatment of each mouse. One control mouse died and 1 anti-α4 integrin mouse was excluded due to a failed ICH surgery.

Antibody Treatment

Mice were treated with IgG2b, κ isotype monoclonal control antibody or anti-α4 integrin antibody (clone R1-2; both low endotoxin and azide-free from Biolegend). Injections were given intraperitoneally, 300 μg/mouse in 300 μl sterile PBS 2-6 hours prior to ICH.

Cylinder Test

At the same time each morning mice were placed into a clean glass jar and observed by a blinded scorer. As mice spontaneously reared and placed forelimbs on the wall of the jar, each rear was scored as right, left, or both for 20 rears. A laterality index was calculated to quantify a lateral forelimb deficit as follows: (right – left) ÷ (right + left + both). A score of +1.0 indicates exclusive use of the right forelimb (left forelimb deficit), whereas a score of 0.0 indicates equal use of the forelimbs and no deficit. Mice were either tested on days 1 and 2 or on days 5 and 7. One mouse from each group failed to sufficiently rear in 20 minutes of observation; they were excluded from cylinder test analyses.

Flow Cytometry

Brain preparation: two or seven days following ICH, mice were sacrificed and intracardially perfused with 40 ml ice-cold PBS. Brains were removed and the ipsilateral hemisphere was mechanically and enzymatically (collagenase/dispase and DNase; Roche) digested into a single-cell suspension. The resulting homogenate was filtered through a 70 μm cell strainer and suspended on a 30%/70% isotonic Percoll gradient (GE Healthcare) and centrifuged at 500 × *g* at room temperature. The interphase was harvested and washed for staining.

Blood preparation: 150 µl of blood were lysed with two room temperature incubations in an ammonium chloride hypotonic solution and centrifuged at 450 × g. The resulting pelleted leukocytes were washed for staining.

Cell staining: all samples were incubated for 10 minutes in Fc block (eBioscience) with rat IgG to block Fc receptors and minimize non-specific antibody staining. Samples were then incubated for 15 minutes with monoclonal antibodies against the following cell-surface markers: CD45, αLβ2/LFA-1 (Biolegend), Ly6G/1A8, Ly6C (BD Biosciences), CD4, CD8, CD62L/L-selectin, CD11b (Tonbo Biosciences), and CD49d/α4 integrin (eBioscience). Dead cells were identified in each sample by their excessive uptake of Alexa Fluor 350-carboxylic acid, succinimidyl ester (CASE; Life Technologies). Counting beads (10,000 per sample; Life Technologies) were added to determine the portion of each sample that was analyzed by the cytometer. The number of beads collected was then used to calculate the total number of cells present in each brain sample and to back-calculate the concentrations of leukocyte populations in the blood. Fluorescence-minus-one (FMO) negative controls were used for each antibody and were made freshly each preparation day. Single-stain positive controls were used for cytometer compensation and an unstained negative control was used to determine background.

Data collection & analysis: cells were evaluated using an LSRII flow cytometer running Diva software (BD Biosciences) equipped with 5 laser lines (355, 405, 488, 561, and 640 nm). Data were analyzed using FlowJo software (Tree Star). As shown in Supplementary Figure I below, only single cells found to be alive by CASE staining were used in analyses. $CD45^{hi}$ cells were considered blood-derived leukocytes in the brain. Cell classification is as follows: $CD45^{hi}$, $CD4^{+}$ (CD4 T cells); $CD45^{hi}$, $CD8^{+}$ (CD8 T cells); $CD45^{hi}$, $CD4^{-}$, $CD8^{-}$, $Ly6G^{+}$, $Ly6C^{+}$ (neutrophils); and $CD45^{hi}$, $CD4^{-}$, $CD8^{-}$, $Ly6G^{-}$, $CD11b^{+}$, $Ly6C^{hi}$ (inflammatory monocytes). In Figure 1 these populations were then analyzed for their surface levels of specific adhesion molecules by the mean fluorescence intensity (MFI), a measure of molecule abundance. The MFI was then multiplied by the proportion of each population that stained positive for that specific adhesion molecule to find the integrated MFI (iMFI) displayed in the Figure 1 bar graphs, which is a combined measure of molecule frequency and surface density.

Statistical Analyses

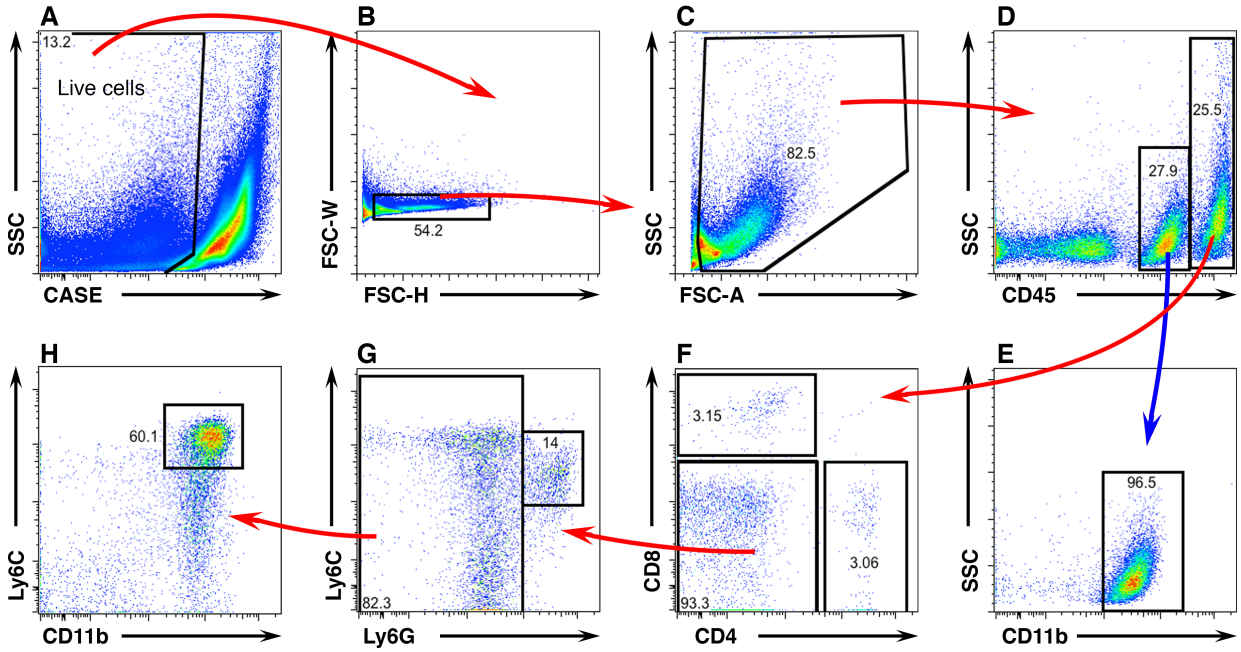
Data were tested for normality using the Shapiro-Francia test at $\alpha=0.05$. Distributions found to be approximately normally distributed were tested for differences using an unpaired *t* test, whereas distributions found not to be normally distributed were tested using the Mann-Whitney *U* test. *P* values less than 0.05 were considered statistically significant. All statistical tests were performed using Stata software.

Supplemental Table

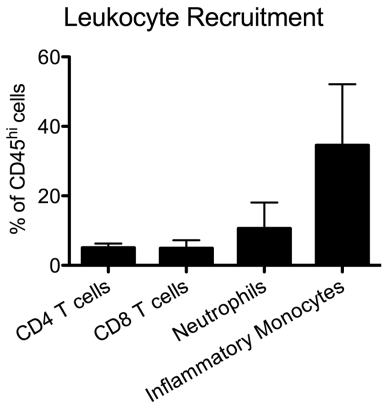
Supplemental Table I:
Mouse physiological variables during ICH surgery were unaffected by pre-treatment with an α4 integrin blocking antibody.

Treatment	Temperature (mean ± SD)	p value temperature	Respiratory Rate (mean ± SD)	p value respiratory rate
Isotype Control	36.54 ± 0.70	0.518	89.42 ± 9.96	0.241
Anti-α4 Integrin	36.40 ± 0.71		94.76 ± 15.23	

Supplemental Figures



Supplemental Figure I. Flow cytometry gating strategy in a day 2 ICH brain. **A**, Cells staining low or negative for CASE were considered live cells. **B**, Single cells, as assessed by forward scatter height (FSC-H) and forward scatter width (FSC-W) were gated for further analysis. **C**, A "leukocyte gate" was drawn on the forward scatter area (FSC-A) by side scatter (SSC) plot to isolate all leukocytes and microglia. **D**, CD45 intensity was examined to separate microglia from blood-derived leukocytes. **E**, $CD45^{low}$ cells were classified as $CD11b^{+}$ microglia. **F**, $CD45^{hi}$ cells were further interrogated for the T cell markers CD4 and CD8. **G**, Blood-derived non-T cells were examined by Ly6G and Ly6C to find double-positive neutrophils. **H**, The remaining cells were then analyzed by CD11b and Ly6C to locate the $Ly6C^{hi}$ inflammatory monocytes. All cell population gates were based on FMO negative controls. The numbers inside or near each gate refer to the percentage of cells on the entire plot that fall within the respective gate.



Supplemental Figure II. Inflammatory monocytes represent the largest population of $CD45^{hi}$ blood-derived leukocytes in the brain 2 days after ICH. Bars indicate mean ± SD. N=4.