Materials and Methods

Animals

Data pertaining to mice from the C3H/FxSJL cross were acquired using histological carotid sections processed from our previous study[1]. Congenic mice were created using marker-assisted selection as originally proposed by Lande and Thompson[2]. We introgressed the chr11 *Im*2 locus[1] from SJL on the C3H/F background. We began by crossing female C3H/F with male SJL mice to produce an F1 generation. Male F1's were bred back with C3H/F females to produce N2 progeny. This pattern was continued until generation N5. The genetically selected males and females produced after generation N5 were intercrossed to develop two *Im*2 chr11 congenic lines, termed C3H/F.SJL.11.1 and C3H/F.SJ.11.2. All males in N2-N5 generations were genotyped to select for those carrying SJL *Im*2 alleles. DNA obtained by tail snip digestion (Promega Wizard DNA extraction kit) underwent polymerase chain reaction (PCR) using a PCR premix (True Allele) based reaction. This was done using fluorescently labeled DNA microsatellite markers present in or flanking the QTL regions to monitor the heterozygosity of the first backcross. The markers were: *D11Mit2.1*, *D11Mit214.1*, *D11Mit231.1*, and *D11Mit289.1* (Applied Biosystems Inc). We assured that our founder congenic mice were on the C3H/F background by whole genome scan as we previously reported[1]. Genotyping data were analyzed using the GeneMapper software.

Surgery

Mice were anesthetized and underwent partial ligation surgery as previously published[1, 3]. To establish the whole genome linkage thresholds, permutation tests were run on our data at 1 cM intervals for 10 000 permutations. Suggestive (P=0.67) and significant (P=0.05) levels were established as suggested[4].

Morphometry Quantification

Analysis of vessel compartment values was done as previously described[3]. Briefly, 14 days following ligation vessels were saline perfused, fixed in 10% neutral buffered formalin and paraffin embedded. Volumes were calculated using hematoxylin and eosin stained sections with MCID Elite 6.0 Imagine System software (Imaging Research Inc).

Linkage Analysis

We used MapManager QTX20b to perform linkage analysis as previously described[1].

$CD45^+$ and α_1-Smooth Muscle Actin (α_1-$actin^+$) Double Immunostaining

We utilized samples from our published C3H/FxSJL backcross for $CD45^+$ and α_1-$actin^+$ double immunostaining. Carotid sections between 600 to 1,200μm below the bifurcation from parental, F1, N2 and congenic mice which were used for immunohistochemistry. Sections were treated with 3% H2O2 for 10min at room temperature followed by antigen retrieval for 20min with steam using 10mM HIER buffer, pH=6.2. For $CD45^+$ staining sections were blocked in 5% rabbit serum followed by overnight incubation at 4°C with rat anti-mouse $CD45^+$ antibody (1:100, Pharmingen) in DAKO antibody dilutent. The negative control antibody was rat IgG2B (1:100, Pharimagen). The secondary antibody was a biotinylated rabbit anti-rat IgG (1:100, Vector). ABC-HRP conjugate (Vector PK-6100) was used in conjunction with the chromogenic HRP substrate/reagent DAB (DAKO) for $CD45^+$ detection. Immunotaining for α_1-$actin^+$ was conducted sequentially to the $CD45^+$ staining. Sections were blocked in 5% horse serum and incubated in mouse anti-human α_1-$actin^+$ antibody (1:1000, DAKO) for 40min at room temperature. The negative control antibody was α_1-$actin^+$, mouse IgG2a (1:1000, DAKO). The secondary

antibody was a biotinylated horse anti-mouse IgG (1:400, Vector). ABC-AP conjugate (Vector AK-5000) was used in conjunction with the chromogenic AP substrate/reagent Vector Red (Vector SK-5100).

Quantification of $CD45^+$ and α_1-$actin^+$ Immunostaining

$CD45^+$ and α_1-$actin^+$ staining were calculated from images of stained vessel sections captured with the Spot software (Diagnostic Instruments) using the 14.2 Color Mosaic Camera (Diagnostic Instruments) in conjunction with the Olympus BX41 microscope. Images were derived from a 40X objective and captured in a 2048x2048 pixel frame. Actual quantification was performed blindly using the Measure Stain tool of the Basic ImagePro software. The areas of interest (AOI) and color intensity thresholds were operator defined. A coefficient of variation of 10% was used for AOI with repeated measurements from the same operator.

Statistical Analysis

Results are reported as mean±SEM. Comparisons for two groups were done using Student *t*-test. Three or more groups were compared by a non-parametric one-way ANOVA. A value of $p<0.05$ was considered statistically significant.

References for Materials and Methods

1. Korshunov VA, Berk BC. Genetic modifier loci linked to intima formation induced by low flow in the mouse carotid. *Arterioscler Thromb Vasc Biol*. 2009;29:47-53
2. Lande R, Thompson R. Efficiency of marker-assisted selection in the improvement of quantitative traits. *Genetics*. 1990;124:743-756
3. Korshunov VA, Berk BC. Flow-induced vascular remodeling in the mouse: A model for carotid intima-media thickening. *Arterioscler Thromb Vasc Biol*. 2003;23:2185-2191
4. Lander E, Kruglyak L. Genetic dissection of complex traits: Guidelines for interpreting and reporting linkage results. *Nat Genet*. 1995;11:241-247.