Supplemental Material

SUPPLEMENTAL METHODS

Cell culture

Human carotid artery smooth muscle cells (CASMC) isolated from 2 healthy donors (organ transplant patient) were grown in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) and 1% of an antibiotic-antimycotic mixture (GIBCO 15240, Invitrogen) and maintained in a humid atmosphere, at 37°C with 5% CO_2. Cells were used between passages 3 and 8 as previously described[1]. Cells were transfected by $CaPO_4$ precipitation with 20 nM siRNA oligonucleotides. Twenty-four hours post-transfection, the cell medium was changed, and 48 h post-transfection, cells were treated as indicated. All siRNAs were obtained from Applied Biosystems. As a control oligonucleotide, 'Silencer Negative Control #2 siRNA' was used. Cells were treated with CML-BSA (Circulex) (1 µg/ml) for different time laps (15, 30, 60 minutes and 48 hours) before analysing.

ELISA assay

CML auto-antibody and direct CML ELISA assays (Circulex) were performed following manufacturer's instructions. Human plasmatic levels of CML-BSA were measured by anti-CML ELISA assay in a blind study was performed on 59 patients. In order to extend these observations, we studied CML plasma levels in a cohort of patients with cardiovascular disease as defined by a history of coronary artery revascularization (60%), ischemic stroke (20%) and symptomatic peripheral vascular disease (33%). Patients were recruited through the outpatient clinics of *Centre Hospitalier Universitaire de Québec – Hôtel Dieu de Québec* Hospital. Blood samples were obtained between 8-10 AM after an overnight fast. The samples were immediately centrifuged and kept at -80°C until analysis. The protocol was approved by the institutional review board and patients provided informed consent.

Confocal microscopy/Immunofluorescence

Rat carotid arteries were fixed with 4% paraformaldehyde. Immunofluorescence was performed on 5 µm carotid slices. CASMC were fixed with 1% paraformaldehyde and permeabilized with 0.2% Triton X-100. P-STAT3 (1/250), RAGE (1/250) antibodies were purchased from Cell Signaling; NFATc1 (1/250) from Abcam. Alexa Fluor (1/1000) 488 and 594 were used as secondary antibodies. For proliferation measurements, PCNA antibodies (1/400) from DAKO and Ki67 antibodies (1/250) from Millipore were used. For apoptosis assays, TUNEL Apoptag kit from Millipore and AnnexinV from Clontech was performed following manufacturer's instructions. FLUO3-AM and TMRM reagents were purchase from Invitrogen and applied at the concentration of 5µM on live cells. Nuclei were stained with diamidino-phenylindole (DAPI, DAKO Cytomation, Carpinteria, CA).

Nuclear translocation assays

To measure the activity of transcription factors STAT3 and NFATc1, a ratio was calculated by dividing the amount of cells that had staining of P-STAT3 and NFATc1 in

the nucleus (colocalisation with DAPI) on the total amount of cells. Ratios were transformed into percentage for statistical analysis.

Immunoblotting

Twenty-five micrograms (25 μg) of protein were loaded on SDS-PAGE (8% wt/vol), and then transferred electrophoretically to PVDF membranes. After blocking, primary antibodies were used: RAGE (1:1000, Cell Signaling), phospho-Akt (1:1000, Cell Signaling), Akt (1:1000, Cell Signaling), phospho-STAT3 (1:1000, Cell Signaling), STAT3 (1:1000, Cell Signaling), NFATc1 (NFAT2, 1:1000, Abcam), Pim1 (1:1000, Cell Signaling) and α-smooth muscle actin (1:300, Santa Cruz). HRP-conjugated secondary antibodies were used (Promega). Expression was normalized to actin to correct for loading differences.

Quantitative real time polymerase chain reaction

RNAs were isolated from CASMC with trizol. Reverse transcription was performed with the High capacity cDNA Reverse Transcription kit from Applied Biosystem. cDNA was used for quantitative RT-PCR Taqman. Taqman primers and probes were all purchased from Applied Biosystems and 18S were used as an internal control.

Carotid artery (balloon) injury model

Male Sprague Dawley (strain 001, Charles River) rats (350g) were used. Under anaesthesia (2% isoflurane), a neck incision was made. A 20 mm section of the left common carotid artery was isolated and temporarily occluded to prevent retrograde blood loss. After proximal arteriotomy, a 2F Fogarty embolectomy catheter was introduced to perform an antegrade balloon inflation along a 15 mm segment of the common carotid artery[2]. The catheter, containing either siSCRM (10μM) or siRAGE (10μM) was maintained in place for 30 minutes to assure efficient transfection. The lumen was then flushed with heparinized saline, the arteriotomy was closed with 9-0 Prolene sutures and the perfusion was restored. Hematoxylin and Eosin (H&E) staining was performed on carotid sections to measure vascular neointima cross-sectional area 14 days post injury (5 rats per group were studied).

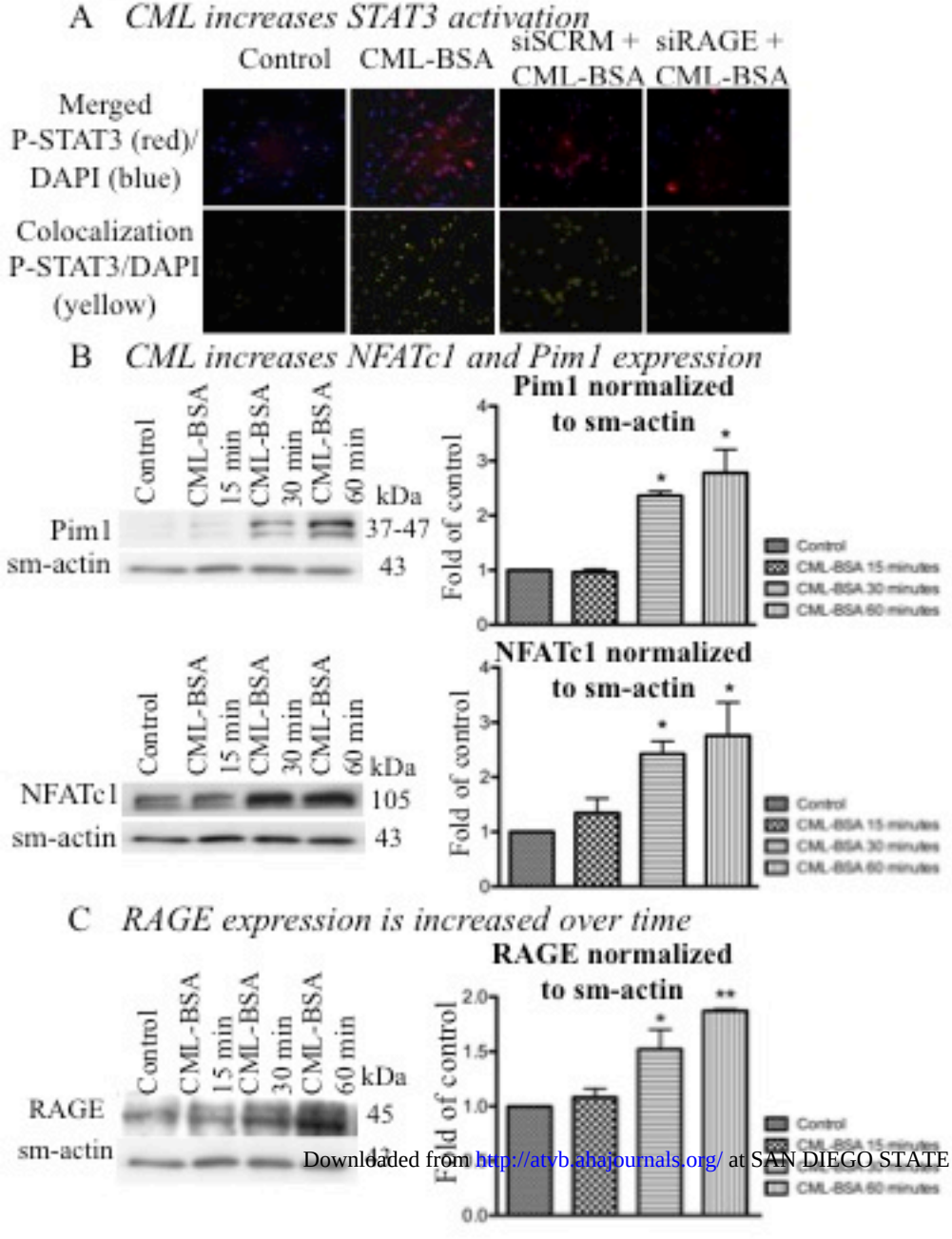
Statistics

Data are presented as mean±SD with Mann Whitney U test for human serum CML levels and mean±SEM for all other studies. Normality of our data was assessed by the Shapiro-Wilk normality test. All our data were normally distributed ($P>0.05$). For comparison between 2 means, we used unpaired Student *t* test. For comparison between 2 means, we used 1-way ANOVA followed by Tukey-Kramer tests. P values less than 0.001 (***), 0.01 (**) and 0.05 (*) were considered as statistically significant.

Supplemental References

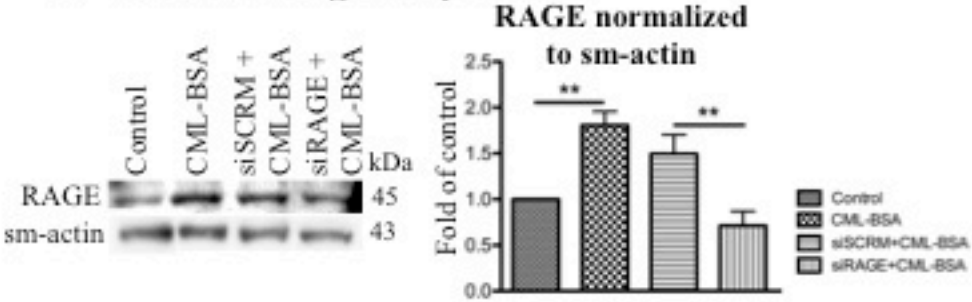
1. Bonnet S, Paulin R, Sutendra G, Dromparis P, Roy M, Watson KO, Nagendran J, Haromy A, Dyck JR, Michelakis ED. Dehydroepiandrosterone reverses systemic vascular remodeling through the inhibition of the Akt/GSK3-{beta}/NFAT axis. *Circulation.* 2009;120:1231-1240.
2. Lambert CM, Roy M, Robitaille GA, Richard DE, Bonnet S. HIF-1 inhibition decreases systemic vascular remodelling diseases by promoting apoptosis through a hexokinase 2-dependent mechanism. *Cardiovasc Res*;88:196-204.

Supplemental Figure I

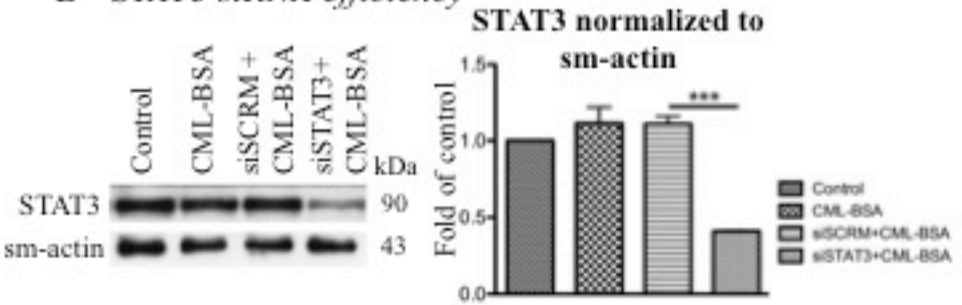


Supplemental Figure II

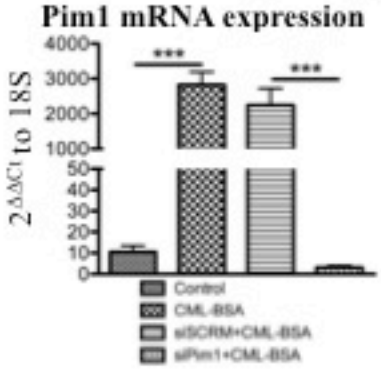
A *RAGE siRNA efficiency in vitro*



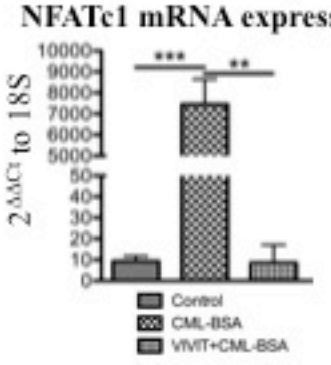
B *STAT3 siRNA efficiency*



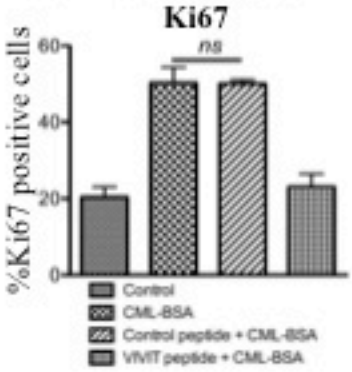
C *Pim1 siRNA efficiency*



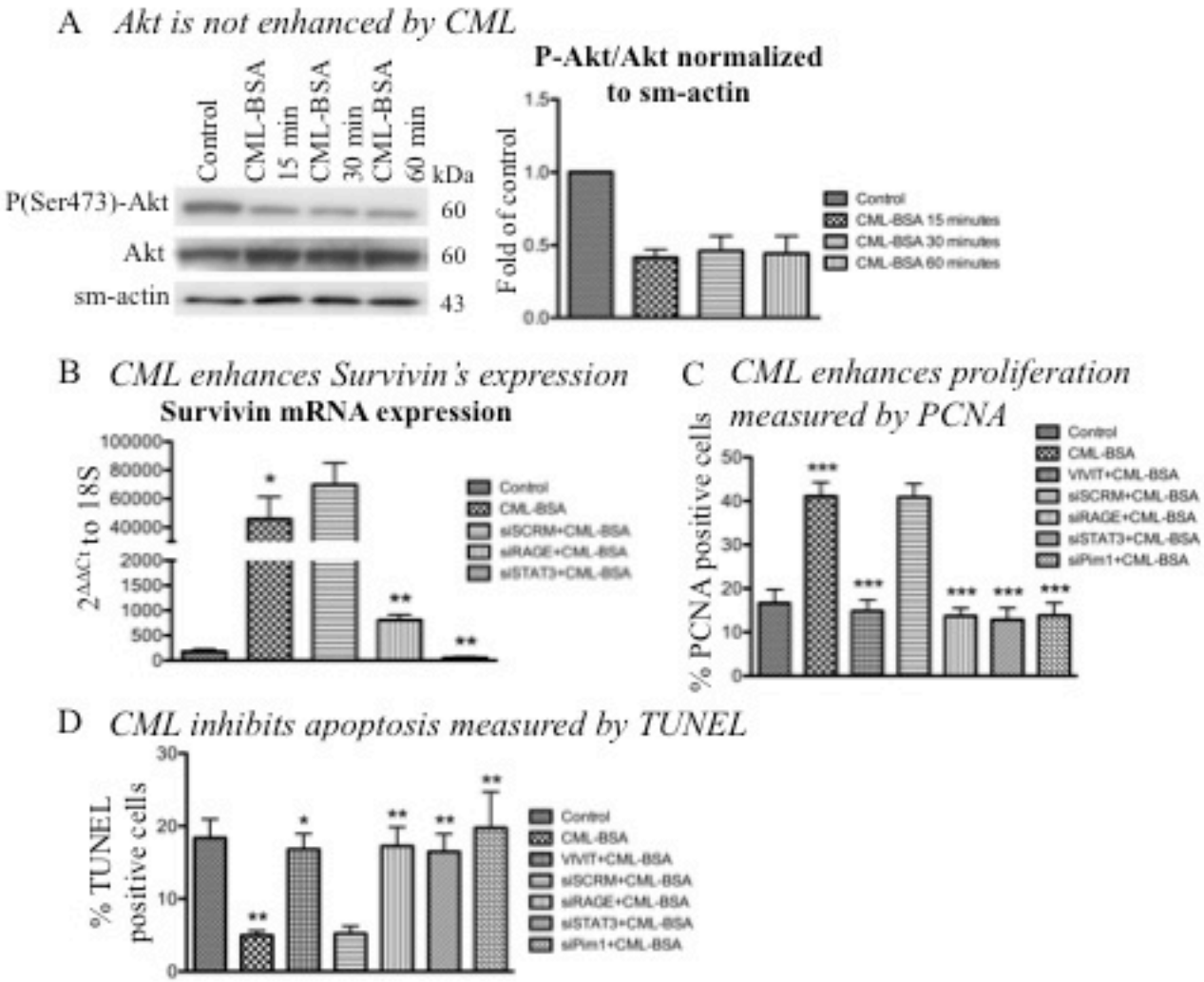
D *VIVIT efficiency*



E *Control peptide does not have any effect*

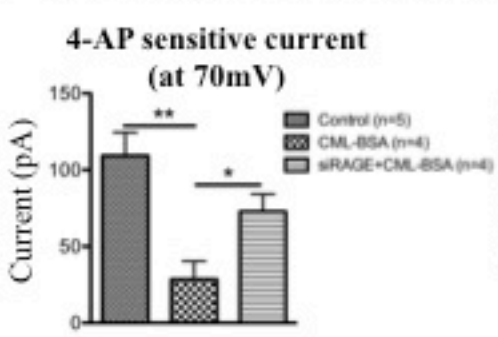


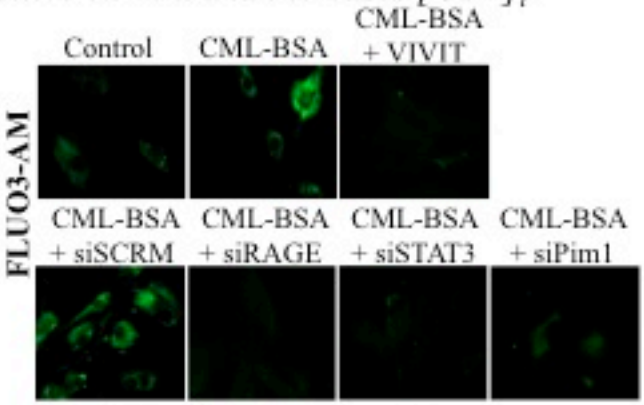
Supplemental Figure III



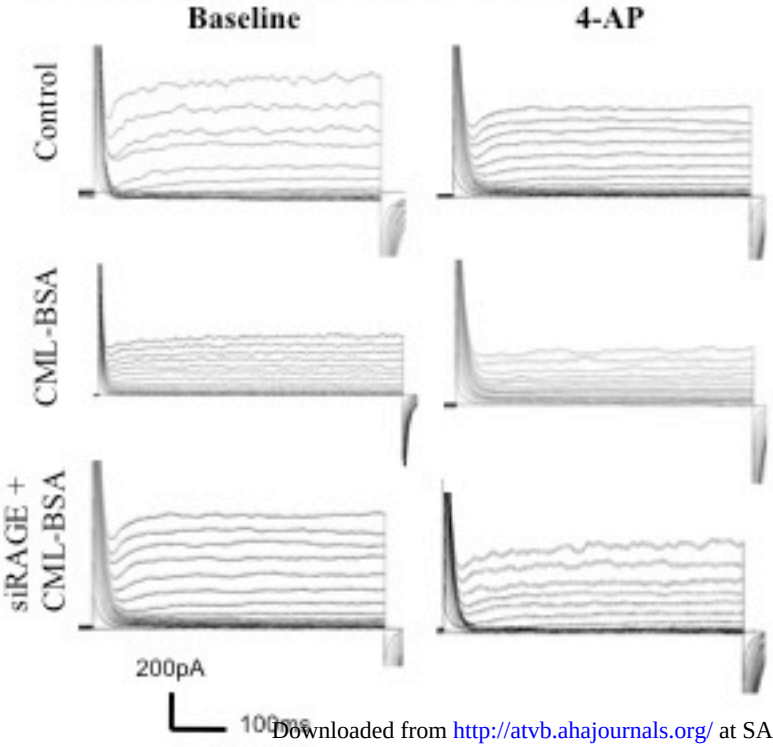
Supplemental Figure IV

A *CML-BSA decreases 4-AP sensitive current and increases $[Ca^{2+}]_i$*



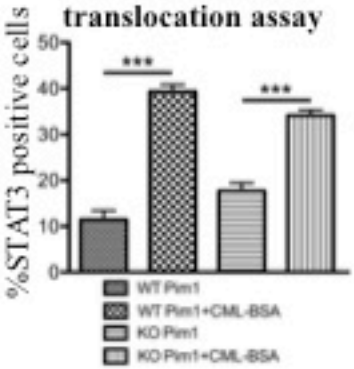


B *Patch clamp representative currents*

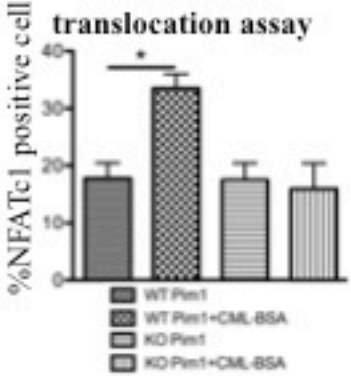


Supplemental Figure V

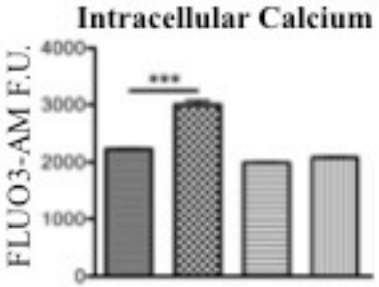
A *STAT3 is activated in K.O. Pim1 mice*

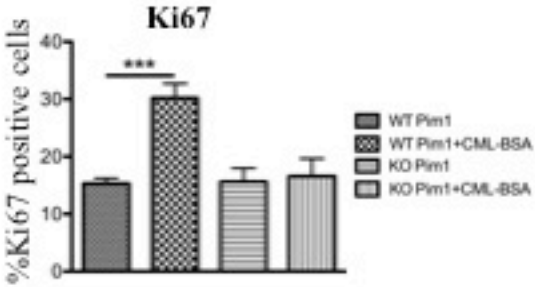


B *NFATc1 is not activated in K.O. Pim1 mice*

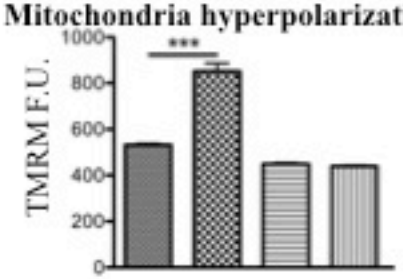


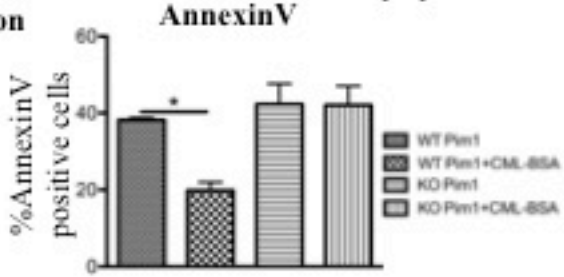
C *K.O. Pim1 mice are resistant to CML-induced proliferation*





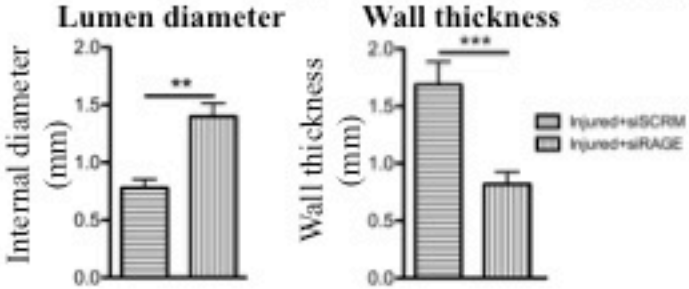
D *CML treated K.O. Pim1 mice are not resistant to apoptosis*



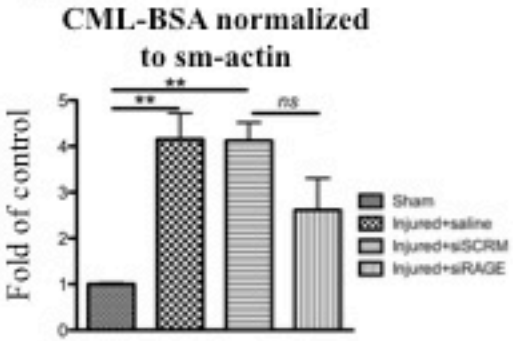


Supplemental Figure VI

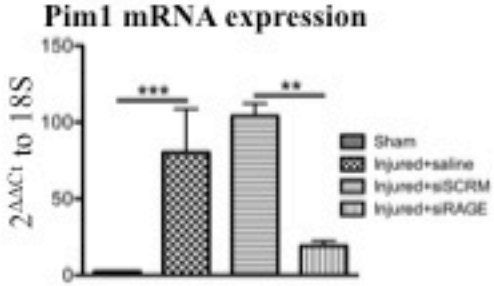
A *Vascular remodeling measured by echography*



B *CML-BSA is increased in injured carotid*



C *Pim1 expression is decreased by RAGE inhibition*



SUPPLEMENTAL FIGURE LEGEND

Supplemental figure I

(A) Representative immunofluorescences of P-STAT3 translocation assay. (B) Pim1 expression and NFATc1 protein activation occur 30 minutes post CML-BSA (1μg/mL) exposure measured by immunoblot (2.4 fold increase; *n=4, p<0.05*). Pim1 and NFAT activation are maintained through time, showed by a 2.75 fold increase after 1 hour. (C) CML-BSA induces a positive feedback loop, increasing RAGE expression over time. In fact, 30 minutes after CML-BSA exposition, RAGE expression is increased and increases more after 1 hour (1.8 fold increase; immunoblot, *n=4, p<0.05*).

Supplemental figure II

(A) RAGE siRNA efficiency is demonstrated by imunoblot. CML-BSA induces a 2 fold increase in RAGE expression and RAGE siRNA reduces its expression of 2.75 fold (*n=3, p<0.01*). (B) STAT3 siRNA efficiency is demonstrated by imunoblot. STAT3 total expression is usually constant, but when cells are treated with STAT3 siRNA, its expression is decreased (2.7 fold decrease, *n=3, p<0.001*). (C) Pim1 siRNA efficiency was measured by qRT-PCR as siPim1 causes a 300-fold decrease in Pim1 expression fold (*n=3, p<0.001*). (D) VIVIT, NFAT's inhibitor peptide, also decreases NFATc1 expression, showed by qRT-PCR, since NFATc1 autoregulates itself (*n=3, p<0.01*). (E) VIVIT peptide is used to block NFAT activation and we confirmed that the control peptide has no effect as cells treated with CML-BSA and cells treated with CML-BSA and the control peptide have the same proliferation rate (*n=3*).

Supplemental figure III

(A) NFATc1 activation by CML-BSA is not due to Akt pathway showed by immunoblot (*n=3*). P-Akt/Akt ratio does not change between cells treated with CML-BSA. Furthermore, this ratio is below the one found in control cells (not treated) (B) CML-BSA enhances Survivin expression through RAGE/STAT3 axis showed by qRT-PCR (*n=3, p<0.01*). Indeed, CML-BSA enhances Survivin expression by 175 fold and RAGE or STAT3 inhibition (by siRNA 20nM) decreases Survivin expression by at least 60 fold. (D) PCNA (proliferating cell nuclear antigen) is another method used to measure cell proliferation. CML-BSA induces an increase of 60% in proliferation (*n=50 CASMC/experiment for 5 experiments, p<0.001*) and when RAGE (siRNA), STAT3 (siRNA) or NFAT (VIVIT 4μM) is blocked, proliferation is restored to normal rates (*n=250, p<0.05*). Proliferation ratio was calculated by dividing positive cells for PCNA (i.e. PCNA in the nucleus) by the total amount of cells (visualised by DAPI) by immunfluorescence. (E) TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) is another method to measure apoptosis and we demonstrated that CML-BSA inhibits apoptosis in CASMC (decrease of 25%) through a RAGE/STAT3/Pim1/NFAT pathway since their blockade restore apoptosis (*n= n=50 CASMC/experiment for 5 experiments, p<0.05*). Apoptosis ratio was measured by diving cells which had TUNEL staining (immunofluorescence) by the total amount of cells (DAPI).

Supplemental figure IV

(A) CML-BSA decreases 4-AP (1mM) sensitive current at 70mV (*n=3, p<0.05*), which corresponds to a decrease of voltage gated Kv channels. Decrease in Kv channels accounts for the increase of intracellular calcium, showed by FLUO3-AM. (B) Representative currents in different conditions using whole cell patch clamping.

Supplemental figure V

(A) CML-BSA stimulates STAT3 activation measured by translocation assay (*n=30 cells per condition per experiment for 3 experiments, p<0.001*) in both wild type and Pim1 K.O. CASMC since STAT3 is upstream of Pim1. (B) Since Pim1 is responsible of NFATc1 activation, Pim1 K.O CASMC treated with CML-BSA (1µg/mL) did not have activated NFATc1 (*n=30 cells per condition per experiment for 3 experiments, p<0.05*). (C) Wild type CASMC treated with CML-BSA have increase calcium, measured by FLUO3-AM (*n=30 cells per condition per experiment for 3 experiments, p<0.001*), and proliferation, measured by Ki67 and K.O Pim1 CASMC are resistant to CML-BSA-induced proliferation (*n=30 cells per condition per experiment for 3 experiments, p<0.001*). (D) Wild type CASMC treated with CML-BSA have hyperpolarised mitochondrial membrane, measured by TMRM (*n=30 cells per condition per experiment for 3 experiments, p<0.001*), and decrease apoptosis and K.O Pim1 CASMC are not resistant apoptosis, measured by AnnexinV (*n=100, p<0.05*).

Supplemental figure VI

(A) By echography, we measured carotid lumen diameter and carotid wall thickness in rats with carotid injury, which had either siSCRM or siRAGE. We demonstrated that RAGE blockade increases lumen diameter (n=5 rats per group, *p<0.01*) and reduces wall thickness (*p<0.001*), which would result in restoring blood flow. (B) By western blot, we demonstrated an increase of CML-BSA in injured carotid (*n=4, p<0.01*). (C) Pim1 mRNA levels in carotid of the different groups were measured by qRT-PCR. Pim1 is increased in injured carotid and showed a 5-fold decreased when RAGE was blocked (*n=5 per group; p<0.01*).