

# Neuroserpin, a thrombolytic serine protease inhibitor (serpin), blocks transplant vasculopathy with associated modification of T-helper cell subsets

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## Summary

Thrombolytic serine proteases not only initiate fibrinolysis, but also are up-regulated in vascular disease and acute inflammatory responses. Although the serine protease inhibitor (serpin) plasminogen activator inhibitor-1 (PAI-1) is considered a main regulator of thrombolysis, PAI-1 is also associated with vascular inflammation. The role of other serpins that target thrombolytic proteases, PAI-2, PAI-3, and neuroserpin (NSP), in vascular inflammation is, however, less well defined. NSP is a mammalian serpin that, similar to PAI-1, inhibits urokinase- and tissue-type plasminogen activators (uPA and tPA, respectively) and has been most closely associated with the nervous system, with a demonstrated protective role after cerebral infarction in mouse models. However, the role of NSP in systemic arterial inflammation and plaque growth is not known. Serp-1 is a myxoma viral serpin that also inhibits tPA and uPA, as well as additionally inhibiting plasmin and factor Xa (fXa). Serp-1 has

proven highly potent anti-inflammatory and anti-atherogenic activity. Here we assess the effects of NSP treatment on plaque growth and T-helper (Th) lymphocyte activity in a mouse aortic allograft transplant model, with comparison to Serp-1. NSP and Serp-1 both significantly reduced plaque growth and T-cell invasion. T-bet (a Th1 differentiation marker) was significantly reduced in transplanted aorta with associated reductions in Th1 and Th17, but not Th2, in splenocytes. NSP had additional Th modifying activity in non-transplanted mice. In summary, this is the first report that NSP possesses anti-inflammatory activity in systemic arteries, modifying Th cell responses and significantly reducing plaque growth in mouse aortic allografts.

## Keywords

Serpin, neuroserpin, T-helper lymphocyte, transplant vasculopathy, atherosclerosis

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## Introduction

Serine proteases in the thrombolytic cascade, urokinase- and tissue-type plasminogen activators (uPA and tPA, respectively), activate plasmin, which drives fibrinolysis. The thrombolytic proteases have both thrombolytic (clot dissolving) and pro-inflammatory actions. Up-regulation of inflammatory responses accelerates development of occlusive plaque in transplant vasculopathy (1, 2) which is associated with chronic rejection and is a leading cause of transplanted organ loss after the first year post transplant (3, 4). Chronic transplant rejection and vasculopathy cause graft loss due to ischaemia and organ scarring. The thrombolytic proteases, their receptors, and the serine protease inhibitors (serpins) act as regulatory counterbalances in haemostasis and vascular inflammation (5, 6). Native vascular serpins that bind to and inhibit thrombolytic proteases, include the plasminogen activator in-

hibitors (PAI), PAI-1, PAI-2, and PAI-3, as well as neuroserpin (NSP). PAI-1 binds tPA, uPA, activated protein C, and thrombin and is considered the major inhibitor of circulating tPA in the blood stream. PAI-1 has proven, but variable, effects in animal models of occlusive vascular disease and aneurysm formation (7, 8). PAI-1 reduces plaque growth in some animal models while other models report accelerated plaque growth and pro-thrombotic activity. Clinically, PAI-1 is considered a marker of increased inflammation based on human trial results. In contrast, PAI-1 has demonstrated consistently protective activity against aneurysm formation in animal models (8). The individual roles for other serpins, specifically PAI-2, PAI-3, and NSP in inflammatory vascular responses are not yet well defined (9, 10).

NSP, is a naturally occurring 46 to 55 KDa mammalian glycoprotein, acting as a functional antagonist of tPA and uPA in the nervous system with secretion from neurons and monocytes. NSP

gene and protein expression are also identified in the pancreas, heart, and pituitary gland, indicating unidentified and potentially broader functions for this serpin outside the nervous system (11). *In vitro*, NSP inhibits tPA and uPA, in order of descending inhibitory activity (12). NSP blocks tPA-dependent seizure propagation, with decreased cell death, reduces stroke size in mouse cerebral infarcts (13), increases motor neuron survival and improves activity in mice with progressive motor neuropathy (a disease associated with increased tPA) (14). Current studies have also investigated the potential of NSP as a therapeutic agent to control tPA activity and prevent haemorrhage after thrombolysis for cerebrovascular accidents (strokes) (11). However, a role for NSP in vascular inflammation and atherosclerosis, and specifically T-cell activation has not been defined.

Large DNA containing viruses also have a wide array of immune modulating functions that block host defences against viral invasion and replication. Serp-1 is a secreted anti-inflammatory myxoma viral protein that inhibits tPA, uPA, plasmin, and factor Xa (fXa), significantly reducing plaque growth and arterial inflammation when administered at picogram to microgram doses (15–17) in animal models. Early Serp-1 treatment reduces acute and chronic allograft and xenograft rejection in rodent aortic, renal, and cardiac transplant models (18–21). Serp-1 also reduces early macrophage and T-lymphocyte invasion, as well as plaque growth in angioplasty and stent implant models. These inhibitory activities are lost in both uPA receptor (uPAR) deficient mouse models and with mutagenesis of the Serp-1 reactive site loop (RSL), indicating that Serp-1 functions in part through modulation of the uPA/uPAR protease pathways (22, 23).

CD4 positive (CD4<sup>+</sup>) Th cellular differentiation into Th subsets is associated with accelerated plaque growth and vascular occlusion (24, 25), but the effects of serpins that regulate the thrombolytic protease pathways on Th cell differentiation, are not known. The Th1 and Th2 subsets are considered central mediators for T-cell inflammatory and immune responses (26). Th1 and associated cytokines are considered pro-inflammatory, while Th2 and its cytokine mediators are considered potentially anti-inflammatory. The T-bet gene is a key factor for Th1 differentiation and Th1 cytokines interferon  $\gamma$  (IFN- $\gamma$ ), tumour necrosis factor  $\alpha$  (TNF- $\alpha$ ) and interleukin-12 (IL-12) are associated with pro-atherogenic activity (27, 28). Conversely, Th2 differentiation and associated cytokines, IL-5 and IL-10, are associated with anti-atherogenic activity (28). IL-4 has both pro- and anti-atherogenic roles. In addition, deletion of the STAT6 (signal transducer and activator of transcription 6) gene, which is important for Th2 differentiation, increases fatty streak development (29). These studies indicate that the Th2 cell subset has a potentially anti-inflammatory role in atherosclerotic plaque development (28).

Th17 is a CD4<sup>+</sup> subset of T-cells that is also associated with immune-mediated diseases including arthritic disorders, such as psoriasis, rheumatoid arthritis, multiple sclerosis, systemic lupus erythematosus (SLE), and non-rheumatic diseases such as asthma and periodontal disease (30, 31). In recent reports, Th17 is also found to have increased expression in patients with atherosclerotic heart disease (32). IL-17 is a Th17 cytokine that also has increased

expression in cardiac and renal allograft transplant models in rodents (33, 34).

The potential regulatory role of NSP in systemic arterial inflammatory responses, outside of the central nervous system, has not been studied. With this work we assess potential natural regulatory functions of NSP in arterial inflammatory responses to injury and during transplant vasculopathy development. Comparative effects of NSP and Serp-1 on Th cell differentiation in response to aortic allograft implants in mouse models are also examined.

## Materials and methods

### Expression and purification of NSP and Serp-1

Neuroserpin protein was expressed in BL21 (DE3) pLysS cells (Invitrogen, Carlsbad, CA, USA). LB media was inoculated with overnight cultures and grown to an optical density (OD) of 0.5<sub>600nm</sub>. Cells were induced with 1 mM IPTG (Isopropyl  $\beta$ -D-1-thiogalactopyranoside) for 3 hours (h), and then pelleted. Cell pellets were resuspended in elution buffer (20 mM Tris + 20 mM Imidazole + 150 mM NaCl) containing EDTA free complete protease inhibitors (Roche) and 2 mM 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF). Samples were French pressed, and the cell debris was pelleted at 15,000 rpm. The supernatant was loaded onto 500  $\mu$ l of cobalt NTA slurry (Sigma-Aldrich, St. Louis, MO, USA). Resin was washed with 100 slurry volumes of His-buffer and eluted with elution buffer. The majority of the NSP localised in the inclusion bodies, however sufficient soluble active protein was expressed (39.5  $\mu$ g/50 ml of culture) so that protein in the inclusion bodies was not used.

Serp-1 protein was kindly provided by Viron Therapeutics Inc. (London, ON, Canada). In brief, Serp-1 was purified from the supernatant of recombinant Chinese hamster ovary (CHO) cell line as previously described (35). Serp-1 purity was over 95% as determined by overloaded Coomassie-stained SDS-PAGE gels and reverse-phase HPLC. Activity of purified NSP and Serp-1 was measured as inhibitory activity for tPA and uPA, using a chromogenic assay (Assaypro, St. Louis, MO, USA) (36).

### General surgical procedure

All research protocols and animal cares conformed to the Guiding Principles for Animal Experimentation and were approved by the local laboratory animal care committees at the University of Western Ontario (London, Canada) and University of Florida (Gainesville, FL, USA). Protein or control saline treatments (0.1 ml/mice, 3/treatment group) were given by intravenous (i.v.) injection (tail vein) immediately after aortic allograft transplant. No increase in mortality was detected for any of the mice after aortic transplant, chemokine injection or with NSP or Serp-1 treatment.

## Aortic allograft transplants

The mouse aortic allograft transplant model has been used previously to assess and identify potential anti-inflammatory therapeutic activity of selected proteins. In this model a donor aortic allograft from PAI-1-deficient (PAI-1<sup>-/-</sup>), untreated mice (C57BL/6 background) are implanted into the aorta of BALB/c wild-type mice (Jackson Laboratory, Bar Harbor, ME, USA) as previously described (37). The aortic allografts develop excessive chronic arterial wall inflammation and neointimal hyperplasia with accelerated plaque growth by the end of four weeks. In brief, using sterile technique, the donor aorta was exposed below the renal arteries through an abdominal incision. A section of infrarenal aorta (PAI-1<sup>-/-</sup>) was removed. Recipient BALB/c mice (n=76) had identical abdominal incisions, the recipient aortic section was isolated by clips and excised, and half of each donor aortic section was transplanted by end-to-end anastomosis into the recipient aorta with 11/0 nylon sutures (Surgical Specialties Corporation, Reiding, MA, USA) (19). Saline (control), NSP, Serp-1 (positive control), or SAA an inactive Serp-1 reactive site mutant (negative control) (37) were injected (6 to 600 ng/g mouse body weight in 0.1 ml saline/mouse) into recipient mice as a single bolus dose immediately after surgery via tail vein. Twenty-two mice with aortic allograft transplants received saline control, 30 transplanted mice were given NSP treatment, 18 mice had Serp-1 treatment, and six mice had SAA treatment (54 mouse aortic transplants in total). Mice were sacrificed at 1 h, 24 h and four weeks post surgery for histology, immunohistochemistry, RT-PCR and flow cytometry (FACS).

## Histological, immunohistochemical, and morphometric analysis

Harvested arterial sections were processed and stained with haematoxylin and eosin as previously described (19, 22, 35). Plaque area, was measured by morphometric analysis by means of the Empix Northern Eclipse trace application program (Mississauga, ON, Canada) using an Olympus CCD colour video camera (Olympus, Orangeburg, NY, USA) attached to the Olympus microscope and calibrated to the microscope objective (22, 35). The mean total cross-sectional area of the intima was calculated for each arterial section. Each allograft specimen isolated from individual mice was cut into three sections and two histology cross sections stained per each of the three allograft section (6 sections per specimen).

Formalin fixed tissue sections were labelled using an ABC kit (Vector Laboratories, Burlingame, CA, USA) as per the manufacturer's protocol. Tissue sections were blocked and labelled for CD3<sup>+</sup> cells using 1:100 primary antibody (rabbit anti-mouse CD3, Abcam, Cambridge, MA, USA), 1:250 biotinylated secondary antibody (goat anti-rabbit IgG, abcam) and avidin biotin complex (Vector Laboratories) as previously described (16, 35, 37). Diaminobenzidine (Sigma-Aldrich) was used for detection and sections were counterstained with haematoxylin. Positively stained cells

were counted in three high power field (HPF) areas in the intimal, medial and adventitial layers of each aortic allograft section analysed.

## Flow cytometry of inflammatory cell responses in mouse ascites

Nine C57BL/6 (PAI-1<sup>-/-</sup>) mice received an intraperitoneal (i.p.) injection of monocyte chemoattractant protein-1 (MCP-1) (50 ng/100  $\mu$ l saline), a CCL2 chemokine, and were simultaneously given an i.v. injection (tail vein) of serpin (Serp-1 or NSP) (60 ng/g mouse body weight – 100  $\mu$ l in saline) or control treatment (3 mice per treatment group). After 18 h, the peritoneum was washed with saline (6 ml) and samples collected. Samples were centrifuged, pelleted, processed and labelled with fluorescein isothiocyanate (FITC)- anti-mouse IFN- $\gamma$  antibody (BD Pharmingen, San Diego, CA, USA) and phycoerythrin (PE)-rat anti-mouse IL-4 antibody (BD Pharmingen) as per manufacturer's protocol. Samples were analysed by fluorescence-activated cell sorting (FACS) Calibur and Cellquest (BD, San Jose, CA, USA). Blood (~200  $\mu$ l) was also collected from each mouse and processed for flow cytometry.

## Flow cytometry analysis of splenocytes

Spleens were isolated from serpin- or saline-treated mice after aortic allograft transplant surgery (PAI-1<sup>-/-</sup> C57BL/6 background to BALB/c recipient) with and without NSP or Serp-1 injection treatment. Mice were sacrificed at 1 h (n=12), 24 h (n=12) and four weeks (n=12) after transplant and serpin or control treatment. The spleen was crushed in a cell strainer (70  $\mu$ m – BD falcon, Bedford, MA, USA), washed with growth media (RPMI-1640 + 1% FBS + 1% penicillin + streptomycin) and splenocytes isolated. RBC lysis was performed on the cell pellet. The splenocytes were washed, re-suspended in growth media and cultured for 18 h before being activated for 6 h with phorbol myristate acetate (PMA) and Ionomycin (Fisher Scientific, Pittsburgh, PA, USA). They were labelled for extracellular CD4-APC (allophycocyanin) and intracellular IL-4-PE, IFN- $\gamma$ FITC, and IL-17-PE monoclonal antibodies as per the manufacturer's (BD Biosciences) protocol. Data acquisition was carried out using FACS calibur for Th1 (CD4<sup>+</sup>/IFN- $\gamma$ <sup>+</sup>), Th2 (CD4<sup>+</sup>/IL-4<sup>+</sup>) and Th17 (CD4<sup>+</sup>/IL-17<sup>+</sup>) and analysed using Cellquest software (BD Biosciences). The data acquisition and analyses was done separately for CD4<sup>+</sup>IL-17<sup>+</sup> cells.

## RT-PCR analysis of aorta transplant specimens

Aorta allograft transplant recipient mice were sacrificed at 1 h, 24 h, and four weeks post surgery/treatment and the transplanted aorta sections were collected in RNAlater (Ambion, Austin, TX,

USA). RNA was isolated from the aorta using RNeasy Mini kit following manufacturer's protocol (QIAGEN, Valencia, CA, USA).

The RNA from the aorta was reverse transcribed to cDNA using Superscript III First Strand kit (Invitrogen) and Real Time PCR was carried out using SYBR Green Core Reagent kit and 7300 RT-PCR system (Applied Biosystems, Austin, TX, USA). The following primers obtained from Sigma-Aldrich were used: GAPDH Forward (F) 5-CTCGCTCCTGGAAGATGGTG-3, Reverse (R)5-GGTG AAGGTCGGTGTGAACG-3; GATA-3(F)5-GAAGGCATCCAGAC CCGAAAC-3, (R)5ACCCATGGCGGTGACCATGC-3; T-BET (F)5-GCCAGGGAACCGCTTATATG-3, (R)5-GACGATCATCTGGGT CACATTGT-3; IL-6 (F)5-GATGCTACAACTGGATATAATC-3, (R)5-GGTCCTTAGCCACTCCTTCTGTG-3; IL-4 (F)5-AGTTGT-CATCCTGCTCTTCTTCTC-3, (R)5-CGACTAATCCATTTGCAT-GATCGTC-3; IFN $\gamma$ (F)5-TGCGCCTAGCTCTGAGACAATGA-3, (R)5-TGAATGCTTGGCGCTGGACCTGTG-3; IL-10 (F)5-GGACT TTAAGGGTACTTGGGTTGCC-3, (R)5-CATTTGATCATCAT GTATGCTTCT-3; IL-17 (F)5-GAAGCTCAGTGCCGCCA-3, (R)5-TTCATGTGGTGGTCCAGCTT-3; and IP-10 (F)5-ACCAT-GAACCAAGTGCTGCCGTC-3, (R)5-GCTTCACTCCAGTTAAG GAGCCT-3.

### Cellular adhesion and core membrane fluidity assays

Cellular adhesion and core membrane fluidity assays were carried out as previously described (38) using PMA as an activator. Each experiment was conducted a minimum of three individual times with five replicates per treatment group for each analysis. Adhesion and fluidity were quantified using calcein fluorescence (excitation 485 nm/emission 527 nm) and 1,3-bis-pyrenylpropane (excitation 320 nm/emission 390 nm and 485 nm), respectively. Fluorescence values from adhesion assays were converted to quantifiable cell numbers using serial dilution as mentioned earlier (38). EnVision<sup>®</sup> Xcite Multilabel Reader (Perkin Elmer, Cambridge, MA, USA) was used for the quantification purpose. The results observed were consistent between each repeated experiment; the best representative data is presented.

### Statistical analysis

Statistical analysis was performed using Statview V5.01 (Cary, NC, USA). The mean plaque area or cell count was calculated from measurements made on the three sections taken from each aortic allograft specimen isolated from each mouse at follow-up (2 histology sections per section, three sections per aortic allograft – 6 sections analysed per mouse). Mean values were used for subsequent statistical analyses. Multiple group comparisons were made using analysis of variance (ANOVA) with Fishers PLSD (Protected Least Significant Difference) and unpaired two tailed Student's t-test for subgroup analysis. P-values less than or equal to 0.05 were considered significant.

## Results

### NSP significantly inhibits plaque growth in mouse aortic allografts

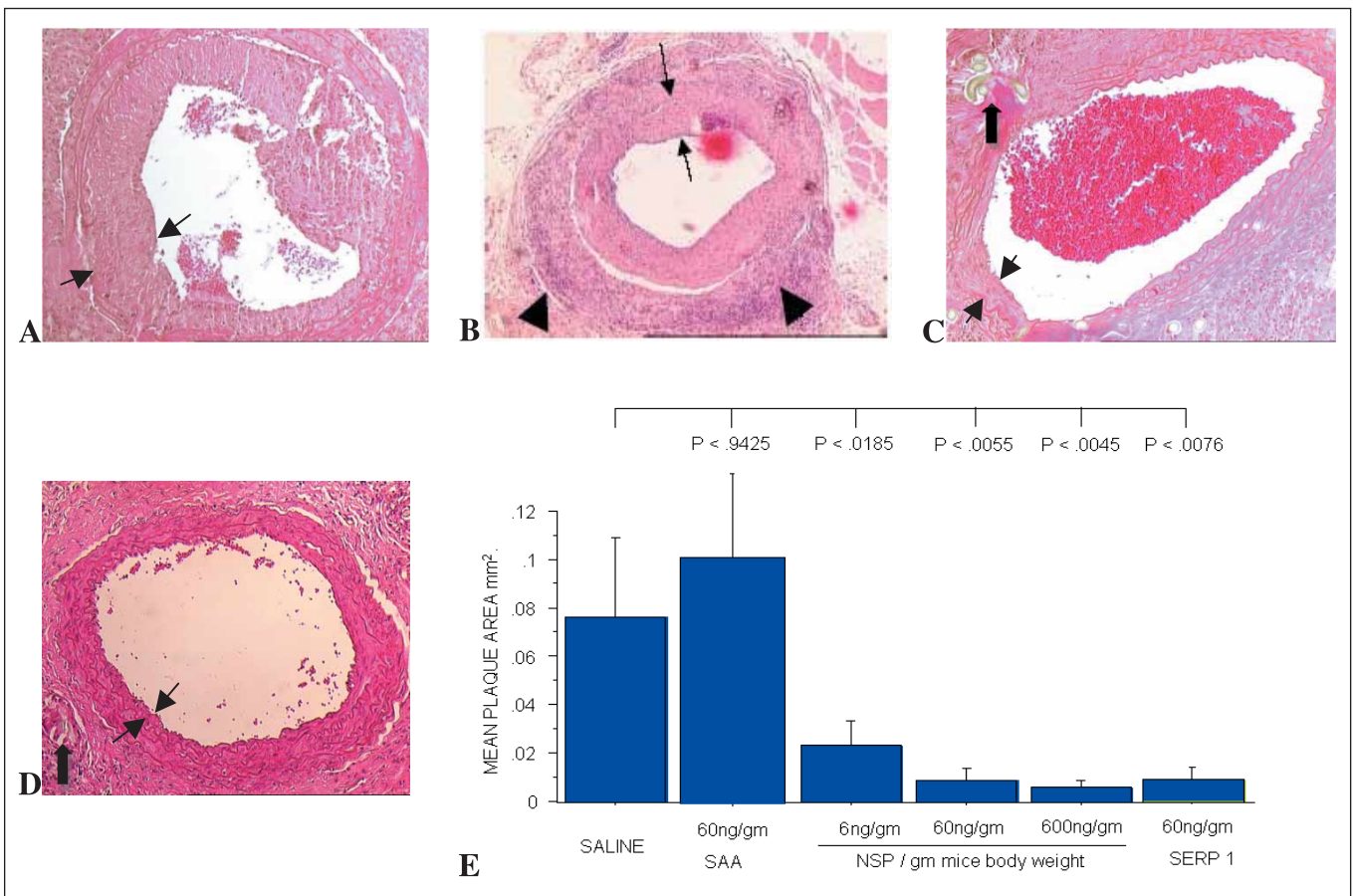
Effects of NSP on neointimal plaque growth and inflammatory cell invasion were initially assessed in the mouse aortic allograft transplant model (n=18 aortic allograft transplants). Effects of NSP are compared to active Serp-1 and SAA that lacks protease inhibitory and anti-inflammatory activity (► Fig. 1). Mice treated with saline (Fig. 1A) or SAA (Fig. 1B) control infusions developed large plaques at the site of aortic transplant with associated inflammatory cell invasion in all arterial layers at four weeks follow-up. NSP treatment, at doses of 6 ng/gm body weight or higher, significantly (P<0.0185) reduced mean plaque area at four weeks follow-up when compared to saline- and SAA-treated mice (Fig. 1C, E).

Inhibition of plaque growth is comparable for NSP and Serp-1 (Fig. 1 C-E). SAA treatment did not significantly alter plaque growth when compared to saline treatment (p=0.9425). No significant increase in thrombosis or mortality is detected in NSP or Serp-1-treated animal groups, consistent with prior studies.

A dose titration analysis (n=18 allograft transplants) indicates a significant dose dependent decrease in plaque area associated with increasing NSP doses (p<0.0185). While the morphometric analysis of mean plaque area per mouse specimen revealed a significant reduction at the lower doses of NSP, analysis of the ratio of intimal to medial thickness which normalises measurements to arterial size, did detect a titratable loss of activity for NSP at the lowest dose (6ng/gm body weight) tested (Saline 2.322 ± 0.681, NSP 600ng/gm 0.497 ± 0.245, P<0.0041, NSP 60ng/gm 0.686 ± 0.261, p<0.0160, NSP 6 ng/gm 1.306 ± 0.389, p<0.0800). When compared to higher doses there is no significant difference in plaque area on comparison between NSP and Serp-1-treated mice at similar doses (Fig. 1E, p=0.3470). There is similarly no significant difference in plaque area for saline or inactive SAA control treatments (Fig. 1E, p=0.9425).

### NSP treatment reduces non-specific mononuclear cell (MNC) and selective CD3<sup>+</sup> T- lymphocyte invasion in aortic allografts

MNC invasion was assessed for non-specific cell invasion on H&E (haematoxylin and eosin) stained histology sections. Invading MNC numbers are significantly reduced in NSP- and Serp-1-treated mice when compared to saline controls (p<0.0001) (► Fig. 2A, B). Reduced MNC infiltration is observed uniformly throughout intimal (Fig. 2A), medial (not shown), and adventitial (Fig. 2B) layers of the aorta, as assessed on aortic cross sections from serpin-treated allograft recipient mice (n=9). This reduction in MNC invasion showed a trend towards reduced efficacy for lower doses of NSP in the dose titration analysis of plaque growth. However, no significant change in MNC infiltration was observed between mice receiving different doses.



**Figure 1: Cross sections of Haematoxylin and Eosin stained aortic allograft transplants isolated from mice four weeks after transplant.** Large areas of intimal plaque growth are detected with inflammatory mononuclear cell invasion in the control saline- (A) and SAA- (B) treated mice. NSP (C) (n=18) and Serp-1 (D) (1.5 µg/mouse, n=6) treatments significantly ( $p < 0.008$ ) reduced plaque area in comparison to controls. Morphometric analysis of plaque area demonstrates a significant reduction in plaque area

with single dose i.v. NSP injection at doses greater than 6 ng/g when compared to saline or SAA controls (n=6). Mean plaque area  $\pm$  SEM is illustrated by bar graphs (E). The small arrows mark the intimal plaque limits. Large arrow heads point to invading mononuclear cells. A suture is seen in the NSP- and Serp-1-treated allograft section (C, D) marked by a larger arrow (Mag. 10X).

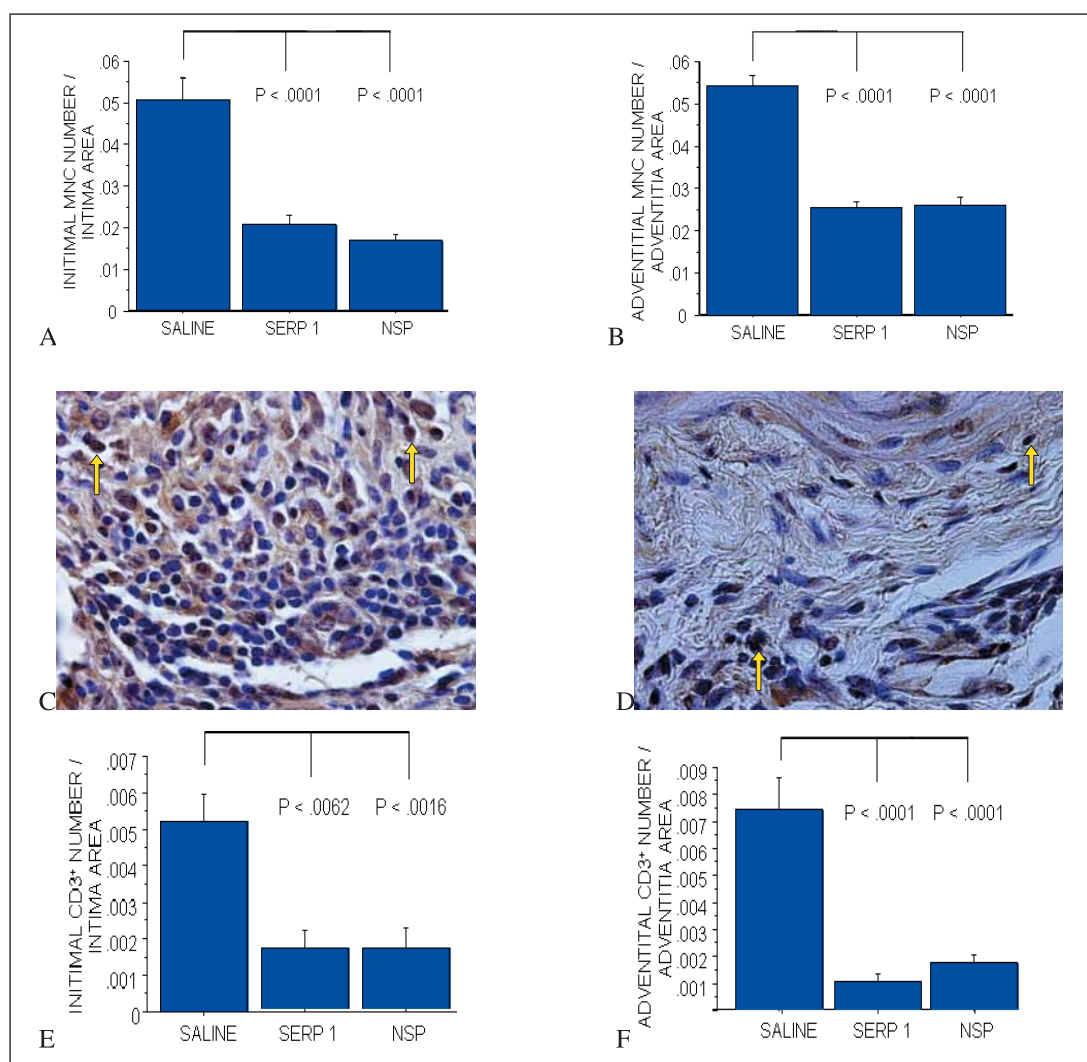
Immunohistochemical analysis of CD3<sup>+</sup> T-cells demonstrates an increase in invading T-lymphocytes in the arterial wall in saline-treated controls (Fig. 2C). NSP or Serp-1 treatment similarly reduced T-cell invasion significantly in treated aortic allograft sections (Fig. 2D-F). As for MNCs, the CD3<sup>+</sup> T-cell count for each arterial layer, intima (Fig. 2E), media (not shown), and adventitia (Fig. 2F), were significantly lower ( $p < 0.0062$ ) after NSP and Serp-1 (Fig. 2E, F) treatment when compared to saline.

### NSP and Serp-1 modulate CD4<sup>+</sup>/IFN- $\gamma$ <sup>+</sup> (Th1) and CD4<sup>+</sup>/IL-4<sup>+</sup> (Th2) subtypes in mouse splenocytes isolated after aortic allograft transplant; FACS analysis

In order to examine potential effects of NSP and Serp-1 treatment on CD4<sup>+</sup> Th cell populations after aortic allograft transplant, we examined Th cell sub-populations in isolated splenocytes using FACS

analysis. At early follow-up time points (1 and 24 h) after aortic transplant, NSP and Serp-1 both significantly reduced total CD4<sup>+</sup> T-lymphocyte populations in splenocytes when compared to saline controls (n=8 allografts for NSP, n=8 for Serp-1, n=8 for saline;  $p < 0.0100$ , ► Fig. 3A-C). This effect is lost at four weeks post surgery. At four weeks, splenocytes from Serp-1-treated mice ( $p < 0.0144$ ) display an increase in CD4<sup>+</sup> cells when compared to saline and there is no significant difference between CD4<sup>+</sup> cell populations in NSP and saline treatments. At early time points (1 and 24 h) Th2/Th1 ratios are increased ( $p < 0.0006$ ) in serpin treatment groups compared to saline (Fig. 3D). This ratio, however, declines with a shift towards increased Th1 at the four weeks time point (Fig. 3E), correlating with the observed increase in Th1 population.

The CD4<sup>+</sup>IFN- $\gamma$ <sup>+</sup> Th1 cell sub-population is significantly ( $p < 0.0003$ ) decreased with either NSP or Serp-1 treatment at 1 and 24 h (Fig. 3F) time points, whereas no significant change is observed in the CD4<sup>+</sup>IL-4<sup>+</sup> labelled, Th2 population (Fig. 3H). At the end of four weeks, the relative percentage of Th1 cells increases in



**Figure 2: Immunohistochemical analysis of aorta cross sections from aortic transplant recipient mice.** Bar graphs illustrate reduced mononuclear cell invasion in the aorta at four weeks post transplant with serpin treatment ( $p < 0.0001$ ) (Intima-A; Adventitia-B). Immunohistochemical staining demonstrates increased numbers of brown stained CD3<sup>+</sup> T-cells (solid yellow arrows) in saline (C) when compared to NSP- (60 ng/gm) (D) treated allografts (Mag. 100X). Reduced CD3<sup>+</sup> T-cell counts per high power field are observed in the intima (E) and adventitia (F) with serpin treatment ( $p < 0.006$ ).

both treatment groups, with NSP having a more significant ( $P < 0.0393$ ) increase than Serp-1 (Fig. 3G). Again, no significant change is observed in the Th2 population.

The FACS data correlates well with immunohistochemistry findings suggesting that the anti-atherogenic activity of NSP and Serp-1 is, at least in part, due to a serpin modulation of T lymphocytes, and specifically Th1 subsets.

### NSP and Serp-1 reduce CD4<sup>+</sup>/IL-17<sup>+</sup> Th17 sub-population differentiation

The same mouse splenocyte cell isolates were examined for effects of NSP and Serp-1 on CD4<sup>+</sup>IL-17<sup>+</sup>, Th17 cell subsets in the mouse aortic transplant model. Both NSP and Serp-1 significantly reduce ( $p < 0.0002$ ) Th17 cell populations at 24 h follow-up when compared to saline controls (Fig. 3I). Serpin treatments do not, however, significantly alter the Th17 splenocyte sub-population at 1 h or at four

weeks post surgery (data not shown), indicating a later onset of effects of serpin treatment on Th17<sup>+</sup> Th cell populations. Both serpins induce similar Th17 regulation patterns at all three time points.

### NSP and Serp-1 alter chemokine-induced cell migration into mouse peritoneal ascites

To assess the effects of NSP and Serp-1 on inflammatory cell responses in mice without aortic transplant surgery (non-operated) mice, cell sub-populations isolated from mouse blood and peritoneal ascites after MCP-1, a CC chemokine, injection into the peritoneal space for stimulation of cell migration were examined by FACS analysis of cells labelled for IL-4<sup>+</sup> and IFN- $\gamma$ <sup>+</sup>. Blood and peritoneal fluid were collected from C57BL/6 mice ( $n = 9$  mice, 3 mice per treatment group) at 18 h post MCP-1 i.p. injection and either NSP, Serp-1, or saline control given by i.v. bolus. FACS analyses of cell isolates from blood samples demonstrated a significant decrease

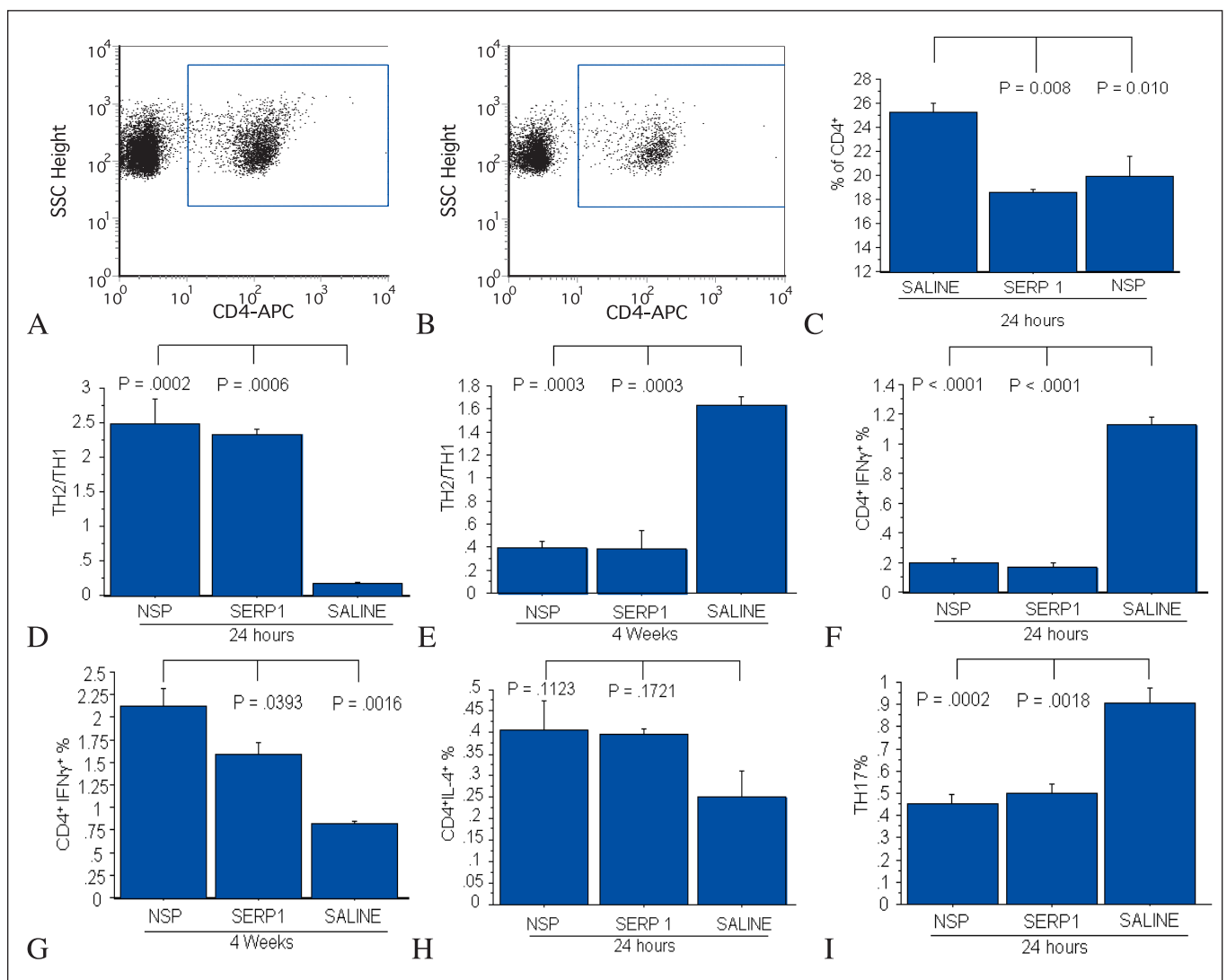
( $p < 0.0042$ ) in total inflammatory cell count in serpin-treated mice when compared to saline controls. Conversely, although the total cell count in peritoneal exudates decreased with serpin treatment, this decrease was not significant (saline=17500, Serp-1=12500, NSP=10000,  $p=0.395$  for Serp-1 and  $p=0.263$  for NSP).

NSP significantly increased the ratio of IL-4<sup>+</sup>/IFN- $\gamma$ <sup>+</sup> when compared to saline treatment, both in peritoneal exudates ( $p < 0.0446$ ) and in blood ( $p < 0.0022$ ) (► Fig. 4A, B). Conversely, Serp-1 treatment did not alter the IL-4<sup>+</sup>/IFN- $\gamma$ <sup>+</sup> (TH2/TH1) ratio in peritoneal exudates ( $p < 0.2820$ ) or blood (Fig. 4A, B) after i.p. chemokine injection in non-transplanted mouse models. The relative percentage of IFN- $\gamma$ <sup>+</sup> and IL-4<sup>+</sup> cell subsets for individual treatment groups

matched the trends in IL-4<sup>+</sup>/IFN- $\gamma$ <sup>+</sup> ratios, but individual changes in IFN- $\gamma$ <sup>+</sup> and IL-4<sup>+</sup> cell counts did not reach significance when compared to saline control treatments (data not shown).

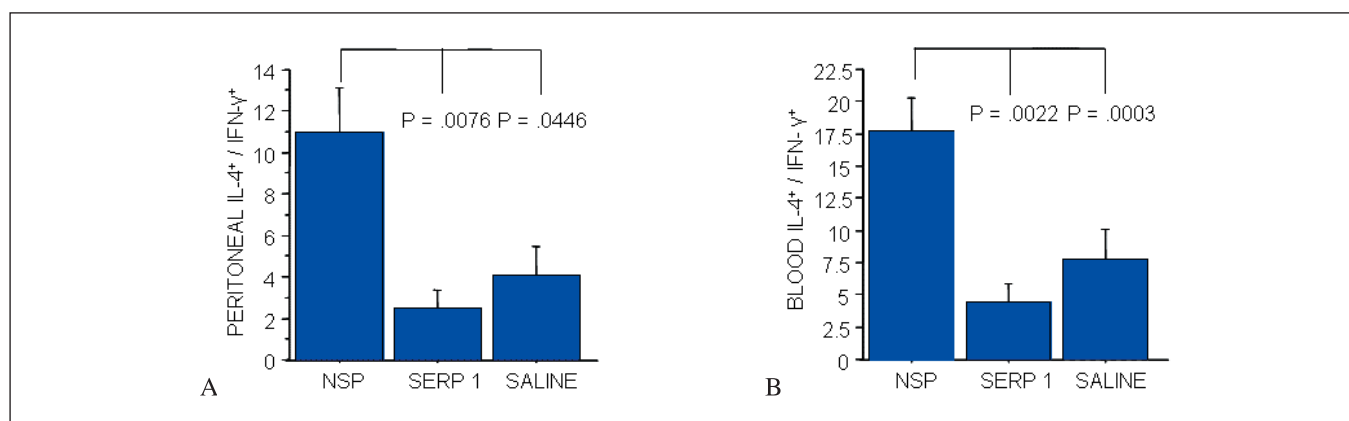
### RT-PCR analysis of aortic allograft sections confirms a reduction in Th1 cell subsets

In order to confirm that changes in Th1, Th2, and Th17 cell subsets detected by FACS in splenocytes were also detected in aortic transplants, quantitative real time RT-PCR analysis of Th1 and Th2 cell



**Figure 3: FACS analyses of Th1, Th2, and Th17 subsets in splenocytes isolated from recipient mice after aortic transplant.** Representative dot plot of CD4<sup>+</sup> T-cells in splenocytes harvested from saline (A) and Serp-1-treated aortic transplant recipient mice (24 h post treatment). CD4<sup>+</sup> T-cells are significantly reduced ( $p < 0.010$ ) at 24 h in Serp-1 (B, C) and NSP (C) treatment mice groups when compared to saline (A, C). The Th2/Th1 ratio is significantly increased 24 h after serpin treatment ( $p < 0.0002$  for NSP and

$p < 0.0006$  for Serp-1) in transplanted mice (D), but decreases at four weeks ( $p < 0.0003$ ) (E). Percentage of CD4<sup>+</sup> IFN- $\gamma$ <sup>+</sup> cells at early time points (1 and 24 h, F) and four weeks (G) correlate with altered Th2/Th1 ratios. The CD4<sup>+</sup> IL-4<sup>+</sup> percentage did not change significantly at any of the observed time points (H). NSP and Serp-1 also reduce the percentage of CD4<sup>+</sup> IL-17<sup>+</sup> Th cells in splenocytes at 24 h (I).



**Figure 4: FACS analyses of IFN- $\gamma^+$  and IL-4 $^+$  subsets in peritoneal fluid and blood from CC chemokine MCP-1 injected mice.** IL-4 $^+$ /IFN- $\gamma^+$  (Th2/Th1) ratio of the cells isolated from peritoneal fluid (A) and blood (B) in non-transplanted mice after i.p. MCP-1 injection demonstrated variable responses to serpin treatment. NSP significantly ( $p < 0.0446$ ) increased IL-

4 $^+$ /IFN- $\gamma^+$  ratios when compared to Serp-1 or saline in the peritoneal fluid (A). Blood from non-transplanted, normal C57BL/6 mice treated with MCP-1 (i.p.) and Serpin (i.v.) also demonstrated increased IL-4 $^+$ /IFN- $\gamma^+$  ratios with NSP ( $p < 0.0022$ ), but not Serp-1 treatment (B).

markers was performed. RT-PCR analysis was used as Th subsets represent a small percentage of invading cells in arterial plaque (► Table 1) making detection and quantification of selected Th subsets difficult on immunohistochemical staining or FACS analysis.

NSP and Serp-1 down-regulated expression of the T-bet gene compared to saline treatment, at 1 h and 24 h time points in aortic allograft isolates, consistent with a reduction in Th1 cell presence, when compared to saline treatment. In both cases the down-regulation by Serp-1 ( $3.1 \pm 0.06$ -fold &  $2.0 \pm 0.7$ -fold for 1 and 24 h, respectively) ( $p < 0.008$ ) is either more pronounced or equivalent to NSP ( $1.55 \pm 0.17$ -fold &  $2.0 \pm 0.31$ -fold) ( $p < 0.037$ , Table 1). However, no significant change is observed at these early time points in expression of GATA-3, a transcription factor important in the development of Th2 lineage (Serp-1,  $p = 0.603$  and NSP,  $p = 0.083$ , data not shown). At four weeks follow-up, no significant change in the expression of T-bet and GATA3 was observed in aortic transplant isolates.

IL-6, a cytokine secreted by T-lymphocytes, is involved in both pro- and anti-inflammatory pathways and is a chief effector molecule for Th17 differentiation (39-41). No significant change in IL-6 gene expression is detected after treatment with either serpin at early time points. However, in the four week follow-up samples Serp-1 significantly ( $p < 0.011$ ) increased IL-6 gene expression by

$7.48 \pm 1.07$ -fold, when compared to saline and NSP treatments. Significant ( $p < 0.024$ ) up-regulation of IFN- $\gamma$  ( $17.54 \pm 4.28$ -fold) and IL-10 ( $29.98 \pm 4.66$ ) expression was also observed at four weeks in Serp-1-treated aortic allograft recipient mice. However these inflammatory regulator genes (28) were not significantly altered at early time points when compared to saline and NSP controls. Unlike Serp-1, NSP does not alter IL-6, IFN- $\gamma$ , or IL-10 gene expression compared to saline controls at any of the observed time points.

The IL-4 gene, is considered a reliable indicator of Th2 differentiation. IL-4 gene expression is not altered at any follow-up time points for any of the treatments in aortic allografts. Similarly, no change in gene expression was observed in the IL-17 or IP-10 gene profile. IL-17 is a cytokine secreted by Th17 and IP-10 is a chemokine secreted by inflammatory cells in the presence of IFN- $\gamma$ .

### NSP and Serp-1 treatment reduce human monocyte and T-lymphocyte activation, *in vitro*

To examine whether NSP and Serp-1 also have the capacity to modify human cellular responses, human cell lines were assayed

**Table 1: Gene fold change between aorta samples of transplant recipient/serpin-treated mice and transplant recipient/saline controls (n=3/treatment group).** Significant differences ( $p < 0.05$ ) between saline and serpin treatment groups are highlighted in bold.

Gene name	Mean fold change compared to saline ( $\pm$ SEM)					
	NSP			Serp-1		
	1 h	24 h	4 weeks	1 h	24 h	4 weeks
T-BET	-1.55 ( $\pm$ 0.17)	-2.00 ( $\pm$ 0.31)	1.02 ( $\pm$ 0.34)	<b>-3.10 (<math>\pm</math> 0.06)</b>	<b>-2.00 (<math>\pm</math> 0.70)</b>	1.75 ( $\pm$ 0.88)
IL-6	1.01 ( $\pm$ 0.34)	2.54 ( $\pm$ 1.22)	1.18 ( $\pm$ 0.23)	1.70 ( $\pm$ 0.21)	5.56 ( $\pm$ 2.28)	<b>7.48 (<math>\pm</math> 1.07)</b>
IFN $\gamma$	1.00 ( $\pm$ 0.44)	3.99 ( $\pm$ 1.68)	2.16 ( $\pm$ 0.82)	-1.33 ( $\pm$ 0.27)	2.03 ( $\pm$ 0.80)	<b>17.54 (<math>\pm</math> 4.28)</b>
IL-10	1.25 ( $\pm$ 0.29)	1.59 ( $\pm$ 1.10)	1.22 ( $\pm$ 0.71)	1.26 ( $\pm$ 0.16)	-2.40 ( $\pm$ 0.48)	<b>29.98 (<math>\pm</math> 4.66)</b>



using Jurkat T-cell and THP-1 monocyte cell lines. Cells were assayed using fluorescent markers for changes in membrane fluidity and cell adhesion *in vitro*. Previous studies from this lab have detected significant shifts in the core membrane fluidity of HUVEC, THP-1, and Jurkat T-cell lines after treatment with various activators (38). In Jurkat T-cells and THP-1 monocytes, core membrane fluidity (Units=lexcimer/Imonomer) is increased with PMA activation, when compared to untreated cells. NSP and Serp-1 both significantly decreased membrane fluidity in PMA-activated Jurkat T-cells (NSP=0.028 ± 0.002, Serp-1=0.028 ± 0.002, Saline=0.033 ± 0.004) ( $p < 0.002$ ) and THP-1 cell lines (NSP=0.031 ± 0.003, Serp-1=0.030 ± 0.002, Saline=0.043 ± 0.003) ( $p < 0.002$ ).

Similarly, when compared to normal cells, PMA activation of THP-1 and T-cells increased cell adhesion (Units=cell numbers) to fibronectin coated (THP-1, normal=37771 ± 18721, PMA=93212 ± 10525; Jurkat T-cells, normal=30849 ± 6161, PMA=55391 ± 9180) ( $p < 0.0013$ ), or uncoated plates (THP-1, normal=33084 ± 14498, PMA=71234 ± 8022; Jurkat T-cells, normal=16152 ± 1659, PMA=26387 ± 3635) ( $p < 0.002$ ), whereas both NSP and Serp-1 treatment inhibit PMA enhanced adhesion of THP-1 and Jurkat T-cells to fibronectin coated (THP-1, NSP=49777 ± 13368, Serp-1=46967 ± 22389 [ $P < 0.0034$ ]; Jurkat T-cells, NSP=41403 ± 4507, Serp-1=32694 ± 11621 [ $p < 0.0351$ ]) or uncoated (THP-1, NSP=38744 ± 10895, Serp-1=43671 ± 14068 [ $p < 0.0075$ ]; Jurkat T-cells, NSP=20367 ± 3860, Serp-1=16789 ± 4980 [ $p < 0.0415$ ]) polystyrene plates. The ability to restore activated human cells toward basal levels of membrane fluidity and cellular adhesion underscores the potency of serpin treatments.

## Discussion

We report here, that mammalian serpin, NSP, significantly inhibits neointimal plaque growth and T-cell invasion in a mouse aortic allograft transplant model, in addition to altering Th cell activation. NSP has previously been closely associated with neuronal cell activation, and anti-inflammatory activity in rodent stroke models, but direct effects of NSP on peripheral arterial inflammatory responses have not been previously described. In the present study the mammalian serpin, NSP, is administered as a single bolus (*i.v.*) immediately after surgery, providing a direct cause and effect relation for NSP and a previously unknown potential activity in regulation of peripheral arterial inflammatory responses.

Serp-1 targets both the thrombolytic pathway, inhibiting tPA, uPA, and plasmin and also the thrombotic pathways, inhibiting fXa. However, as prior work with Serp-1 has demonstrated a requirement for the uPA receptor (uPAR) in mouse models of aortic transplant (22), this would suggest that the uPA/uPAR complex is the main target for Serp-1. PAI-1 has also been reported to alter vascular inflammation and plaque growth but again targets both thrombolytic and thrombotic proteases (7). In contrast, NSP inhibits tPA and uPA exclusively providing a comparator for the effect of inhibition of the plasminogen activators and a potential role in vascular inflammation. By comparing the effects of NSP and

Serp-1 in the aortic allograft and peritoneal ascites models, we differentiate effects of inhibition of the thrombolytic proteases alone (NSP) with the combined inhibition of thrombolytic and thrombotic protease (Serp-1). Given the similar effects of NSP and Serp-1 on allograft neointimal hyperplasia, these findings suggest that the thrombolytic proteases represent central targets for inhibition of allograft inflammation and vasculopathy in mice. However, one must note, that while we differentiate the thrombotic and thrombolytic pathways to some extent, we have not in this study directly assessed the effects of serpins that selectively target factor X and thrombin, *e.g.* the thrombotic pathways. We have tested an inactive mutant of Serp-1, SAA, demonstrating a loss of inhibitory binding to tPA and uPA and also demonstrating a loss of anti-inflammatory activity *in vivo* in the aortic transplant model. We have not however, tested an inactive neuroserpin mutant and thus there is the possibility that these serpins are functioning through an alternative pathway (42). Another limitation of the current study is that it does not address potential anti-inflammatory activity of peptides that might be generated *in vivo* from the tested serpins.

NSP has been found to reduce neuronal damage, macrophage invasion and cerebral infarction size in animal models. Prior work has not examined a potential role for NSP as a regulator of inflammatory cell responses in the circulation throughout the cardiovascular system. Recent work, as noted in the introduction has detected NSP expression in other organs, but a regulatory role for NSP in the peripheral circulation and in inflammatory vascular disease has not been examined. While we have detected a significant inhibitory action for exogenous NSP infusions, we have not here demonstrated that NSP has a role as a native arterial regulatory factor in the systemic circulation outside of the central nervous system (CNS). Further studies will be required to examine this potential role for NSP. Although both NSP and Serp-1 inhibit uPA and tPA, excess thrombosis was not detected within the transplant models. This lack of adverse effects may be attributed to the short circulating half life, the smaller infusion doses given, the relatively low inhibitory activity, or a previously unknown additional activity associated with these serpins.

Although NSP was reported to reduce macrophage invasion after cerebral stroke in mouse models and Serp-1 has been previously demonstrated to alter both macrophage and T-cell invasion after angioplasty or transplant, selected effects of serpins that inhibit thrombolytic proteases, on CD4<sup>+</sup> Th cell differentiation and activation have not been examined. While a decreased infiltration of CD3<sup>+</sup> T-cells is detected in all three layers of the aorta together with an early and significant reduction in Th1 cell subset differentiation in isolated spleen cells in mice after aortic transplant (24 h), we have not demonstrated a direct effect on Th cell subsets in the aorta. The cell numbers infiltrating the aorta, while detectable overall, are not large enough for detection of Th cell subsets by the immunostaining techniques used. However, analysis of gene expression did demonstrate a significant reduction in T-bet—a factor necessary for Th1 differentiation. As noted, there was no significant effect on Th2 in the transplant models indicating a more direct effect of NSP and Serp-1 on the Th1 cell subset

(43). Detection of activated CD4<sup>+</sup> T-cells would have been optimal but is problematic due to the small numbers of CD4<sup>+</sup> T-cells in aortic grafts (Fig. 2). The reduction in CD4<sup>+</sup> T-cells detected in spleen cell isolates during the early inflammatory response did correlate well with late attenuated plaque development and with decreased expression of Th1 specific genes (T-bet) in aortic isolates.

The effects of each serpin on Th cell differentiation were consistently detected in the transplant model, but were not detected in the ascites model, which uses normal mice with chemokine injection. This study comparison suggests that the two serpins modify Th1/Th2 responses under the highly inflamed conditions associated with aortic allograft transplant, but not consistently in normal mice. This Th cell response to serpin treatment with an early increase in the Th2/Th1 responses after aortic transplant is limited in duration, reverting to normal at four weeks. The effects on Th cell differentiation was potent in that a single bolus injection (post transplant) of either NSP or Serp-1 at ng to µg dose levels was capable of altering Th cell response in the first 24 h and inhibiting plaque growth at four weeks.

To assess local T-cell responses in the aortic transplants, gene expression for T-cell markers T-bet and GATA3 in aorta was also assessed. T-bet is a member of the T-box family of transcription factors that regulates Th1 differentiation from naïve Th0 cells (44). Similarly GATA3 transcription factor induces Th2 differentiation (45) from Th0 cells. T-bet gene expression in the recipient aorta was reduced in aortic allograft sections with no significant difference in Th2 (GATA3) responses. This suggests a reduction in the capacity of Th0 cells to differentiate to the Th1 subset, a result that correlates well with the FACS Th1, Th2 analysis of splenocytes during early time points.

Similar to Th1, Th17 and its cytokine IL-17 are also pro-inflammatory and implicated in atherosclerotic plaque aggravation and destabilisation. Th17 related IL-23 cytokine exists as a heterodimer with the Th1 related cytokine IL-12 (46). Hence it becomes necessary to separate out the activity of Th1 from Th17. Latest reports also indicate that Th17 cells, under suitable conditions, can shift towards Th17/Th1 type cells that secrete IFN-γ. Hence it becomes imperative to study and exclude the effect of Th17 population to confirm the influence of Th1 subtypes on inflammation in this transplant vasculopathy. Splenocytes from NSP- and Serp-1-treated aortic allograft transplant recipient mice also demonstrated a decrease in the Th17 subset at 24 h, but not at 1 h or four week time points. Gene expression analyses in the aortic samples do not, however, demonstrate a significant change in IL-17 gene expression, suggesting that the effects on Th1 differentiation occur first and may have a predominant role, suggesting that reduction of the Th17 response is a secondary response with less impact than the reduction in the Th1 cell subsets on vascular inflammation and atherogenesis. Similarly, IL-6 gene analysis does not correlate with the observed Th17 shift. IL-17 a cytokine secreted by Th17 has been associated with kidney rejection in rodents and humans and treatment with the corresponding receptor protein, IL-17R protein, has also been shown to prolong cardiac allograft survival in rodents (30). Thus a reduction in Th17 subsets observed in this model may contribute to the decrease in arterial inflammation and plaque growth.

Although IL-6 and IL-10 gene expression are not altered at early time points, they are both up-regulated by Serp-1 in the aorta of transplant recipient mice at four weeks. The role of IL-6 in inflammation is ambiguous. IL-6 is secreted by macrophages and smooth muscle cells and is also important for Th17 differentiation. Though predominantly pro-inflammatory IL-6 also enhances anti-inflammatory IL-10 cytokine secretion in activated human CD4<sup>+</sup> T-cells (47). Our studies demonstrate that Serp-1-treated transplant recipient mice from the four weeks time point group have increased IL-10 gene expression in the aorta which has anti-inflammatory functions.

Reduced activation of human cell lines, THP-1 (monocytes) and Jurkat T-cells, are also detected after serpin treatment *in vitro* in membrane fluidity and cell adhesion experiments. Cell membranes play a significant role in cell signalling, cellular activity and activity of protein on cell surfaces (48, 49). Altered membrane fluidity is reported in several activated disease states, including atherosclerosis (50). The studies examining cell activation using the membrane fluidity assay are also confirmed in this work by detection of inhibition of THP-1 monocyte and Jurkat T-cell adhesion in response to PMA activation, indicating a potential for anti-inflammatory activity in human cells.

We report here that the mammalian serpin, neuroserpin, actively blocks vascular inflammatory cell responses and neointimal plaque growth in a mouse aortic allograft transplant model. We also demonstrate that two serpins, NSP and Serp-1, significantly reduce inflammatory cell activation and invasion with associated modification of Th cell differentiation after aortic transplant—suggesting the capacity to reduce occlusive transplant vasculopathy development in mouse models.

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