TSG-6: AN INDUCIBLE MEDIATOR OF PARACRINE ANTI-INFLAMMATORY AND MYELOPROTECTIVE EFFECTS OF ADIPOSE STEM CELLS

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Matthias A. Clauss, Ph.D.
DEDICATION

This work is dedicated to my wife Ru, my parents Haiyuan and Yue, and my mentor Keith March and his wife Sarah.

To my beloved wife Ru, thank you for indulging my obsession with research and endless hours in the lab. With my less than minimum income, I cannot imagine the stress, difficulties, and frustrations you have fought against and overcome throughout the years to make ends meet, and make our home a harbor of comfort when I needed it most. Your unconditional love is the fire in my heart that keeps me warm on rainy days and gives me strength and courage to stand up against any challenge in front.

To Mom and Dad, thank you for instilling in me the faith in hardworking, honesty, and self-discipline, and for supporting my pursuit of a wildest dream. I know how hard it is to see other people’s children at home with the family during spring festival while your only son is thousands of miles away. I want you to know you are also part of this achievement.

To Sarah, with six children, two horses, two dogs, one cat in the house, you must have used some magic so that Keith could be there when I needed his guidance most. To Keith, I truly enjoyed those sleepless nights in your basement before deadlines and early morning discussions at Starbucks. Thank you for caring not only my research but also me as a person, and for always respecting, encouraging, and supporting my interest, ideas, and decisions.
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I also thank American Heart Association and Cryptic Masons Medical Foundation for generously supporting my research at this hard economic time.

I also feel blessed to have met all the friends in Indy over the years, whose companionship I thoroughly enjoyed. Your friendship has fulfilled my life with plenty of joy, happiness, and thankfulness.
ABSTRACT

Jie Xie

TSG-6: AN INDUCIBLE MEDIATOR OF PARACRINE ANTI-INFLAMMATORY AND MYELOPROTECTIVE EFFECTS OF ADIPOSE STEM CELLS

Tumor necrosis factor-induced protein 6 (TSG-6) has been shown to mitigate inflammation. Its presence in the secretome of adipose stem/stromal cells (ASC) and its role in activities of ASC have been overlooked. This thesis described for the first time the release of TSG-6 from ASC, and its modulation by endothelial cells. It also revealed that protection of endothelial barrier function was a novel mechanism underlying the anti-inflammatory activity of both ASC and TSG-6. Moreover, TSG-6 was found to inhibit mitogen-activated lymphocyte proliferation, extending the understanding of its pleiotropic effects on major cell populations involved in inflammation.

Next, enzyme-linked immunosorbent assays (ELISA) were established to quantify secretion of TSG-6 from human and murine ASC. To study the importance of TSG-6 to specific activities of ASC, TSG-6 was knocked down in human ASC by siRNA. Murine ASC from TSG-6−/− mice were isolated and the down-regulation of TSG-6 was verified by ELISA. The subsequent attempt to determine the efficacy of ASC in ameliorating ischemic limb necrosis and the role of TSG-6, however, was hampered by the highly variable ischemic tissue necrosis in the BALB/c mouse strain.
Afterwards in a mouse model of cigarette smoking (CS), in which inflammation also plays an important role, it was observed, for the first time, that 3-day CS exposure caused an acute functional exhaustion and cell cycle arrest of hematopoietic progenitor cells; and that 7-week CS exposure led to marked depletion of phenotypic bone marrow stem and progenitor cells (HSPC).

Moreover, a dynamic crosstalk between human ASC and murine host inflammatory signals was described, and specifically TSG-6 was identified as a necessary and sufficient mediator accounting for the activity of the ASC secretome to ameliorate CS-induced myelotoxicity. These results implicate TSG-6 as a key mediator for activities of ASC in mitigation of inflammation and protection of HSPC from the myelotoxicity of cigarette smoke. They also prompt the notion that ASC and TSG-6 might potentially play therapeutic roles in other scenarios involving myelotoxicity.

Keith L. March, M.D., Ph.D., Chair
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<tbody>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>AP1</td>
<td>activator protein-1</td>
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<tr>
<td>APP</td>
<td>acute phase protein</td>
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<tr>
<td>ASC</td>
<td>adipose stem / stromal cells</td>
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<tr>
<td>BFU-E</td>
<td>erythroid burst-forming unit</td>
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<td>BMSC</td>
<td>bone marrow stromal cells</td>
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<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>CFSE</td>
<td>carboxyfluorescein diacetate succinimidyl ester</td>
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<tr>
<td>CFU</td>
<td>colony forming unit</td>
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<tr>
<td>CFU-GEMM</td>
<td>granulocyte, erythroid, monocyte, megakaryocyte colony-forming unit</td>
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<tr>
<td>CFU-GM</td>
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<tr>
<td>CLP</td>
<td>common lymphoid progenitor</td>
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<tr>
<td>CMP</td>
<td>common myeloid progenitor</td>
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<tr>
<td>COPD</td>
<td>chronic obstructive pulmonary disease</td>
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<td>CRP</td>
<td>C-reactive protein</td>
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<td>cigarette smoke extract</td>
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<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
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<tr>
<td>ELISA</td>
<td>enzyme-linked immunoabsorbent assay</td>
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<tr>
<td>FAL</td>
<td>femoral artery ligation</td>
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<tr>
<td>FBS</td>
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<tr>
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<td>GM-CSF</td>
<td>granulocyte macrophage-colony stimulating factor</td>
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<td>granulocyte/macrophage progenitor</td>
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<td>glucocorticoid response elements</td>
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<td>graft versus host disease</td>
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HBSS  Hank’s Balanced Salt Solution
HGF  hepatocyte growth factor
HIA-G5  human leukocyte antigen-G5
HLA  human leukocyte antigen
HO1  haem oxygenase-1
HPC  hematopoietic progenitor cells
HRP  horseradish peroxidase
HSC  hematopoietic stem cell
HUVEC  human umbilical vein endothelial cells
ICAM  intercellular adhesion molecule
IDO  indoleamine 2,3-dioxygenase
IRF  interferon regulatory factors
LSK  Lin-Sca1+c-Kit+
M-CSF  macrophage-colony stimulating factor
MHC  major histocompatibility complex
MMP  matrix metalloproteinase
MNC  mononuclear cell
MPP  multi-potent progenitor
MSC  mesenchymal stem cells
NFIL6  nuclear factor interleukin 6
NO  nitric oxide
NOD/SCID  nonobese diabetic/severe combined immunodeficient
NOS  nitric oxide synthase
PBF  phosphate buffered formalin
PBS  phosphate buffer saline
PCR  polymerase chain reaction
PECAM  platelet endothelial cell adhesion molecule
PGE  prostaglandin E
PGE2  Prostaglandin E2
PMN  polymorphonuclear cells
PPACK  Phenylalanyl-L-Prolyl-L-Arginine Chloromethyl Ketone
<table>
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<td>stem cell factor</td>
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<tr>
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<td>standard error of mean</td>
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<td>transforming growth factor-β1</td>
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<tr>
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<td>tumor necrosis factor</td>
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<tr>
<td>TSG</td>
<td>TNF-induced protein 6, TNFIP6</td>
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<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
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Chapter 1. Introduction

1.1 Inflammation and Mesenchymal Stem Cells

Overview of Inflammation

The inflammatory cascade begins with proinflammatory cytokine release from injured cells or antigen-presenting cells (macrophages or natural killer cells). These cytokines stimulate parenchymal cells to produce chemokines that recruit neutrophils and monocytes. Infiltrated neutrophils not only phagocytize pathogens and cell debris, but also release toxic proteases and free radicals. If this innate immunity does not subside timely, it may cause excessive collateral tissue damage. Monocytes give rise to macrophages, which engulf a large number of viruses and bacteria. Antigens presented by macrophages or NK cells activate T lymphocytes to proliferate. Cytotoxic T cells are responsible for eliminating foreign cells or cells infected with viruses. The proliferation of cytotoxic T cells can be further enhanced by helper T cells. It has been increasingly recognized that excessive or nonresolving inflammation contributes to the damage wrought by degenerative diseases such as atherosclerosis, obesity, diabetes, COPD, and arthritis.1-3

BMSC vs. ASC

Mesenchymal stem cells (MSC) are a heterogeneous population of cells that proliferate in vitro as plastic-adherent cells, have fibroblast-like morphology, form colonies in vitro and can differentiate into bone, cartilage and fat cells.4 Stromal cells that fulfill these criteria have been isolated from almost every type of connective tissue, including bone marrow, adipose tissue, placenta, and umbilical cord.5 Bone marrow-derived MSC (BMSC) were discovered first and are the best characterized type of MSC. Adipose tissue has been identified as a key promising alternative source for MSC, because adipose stem or stromal cells (ASC) can be readily isolated in considerably larger amounts.6 As mesenchymal stem cells, BMSC and ASC share many biological characteristics, and have both been proven effective in a largely overlapping spectrum of disease applications.7-10 That said, MSC residing in these two distinctive niches also have many
differences in their immunophenotype, differentiation potential, transcriptome, proteome, and immunomodulatory activity. For instance, ASC suppressed pokeweed mitogen-induced Ig production from peripheral blood mononuclear cells to a much greater extent than BMSC.$^{11}$ ASC also outperformed BMSC at stimulating the secretion of the immunosuppressive cytokine IL10 by dendritic cells.$^{12}$ The molecular mechanisms underlying these tissue-specific differences, however, remain unknown.

Modulation of Immune Cells by MSC

MSC are described as immune privileged cells, due to low expressions of class II Major Histocompatibility Complex (MHC-II) and costimulatory molecules.$^{13}$ They also interfere with various pathways of the immune response by means of direct cell-to-cell interactions and soluble factor secretion. This is probably mediated by the multiplicity of immune mediators in the secretome of MSC (Figure 1, adapted from reference 14). In vitro, MSC inhibit cell proliferation of T cells, B-cells, natural killer cells (NK) and dendritic cells (DC), producing what is known as division arrest anergy.$^{15}$ Moreover, MSC can stop a variety of immune cell functions: cytokine secretion and cytotoxicity of T and NK cells; B cell maturation and antibody secretion; DC maturation and activation; as well as antigen presentation. It is thought that MSC need to be activated to exert their immunomodulation activities.$^{16}$ In this scenario, an inflammatory environment seems to be necessary to promote their effects; and some inflammation-related molecules such as TNFα have been implicated. It has been observed that MSC recruit regulatory T lymphocytes to both lymphoid organs and the graft.
Figure 1. Multiple proposed mechanisms of interactions between MSC and cells of the innate and adaptive immune systems. 

Literature from multiple groups has shown sophisticated immuno-modulatory effects of mesenchymal stem cells (MSC) via either physical contact or trophic factors. However, endothelial cells, as fundamental structural blocks of blood vessels and barriers against leukocyte extravasation, have not been taken into account. Adapted from reference 14.
Mediators of Immunomodulation by MSC

There is great controversy concerning the mechanisms and molecules involved in the immunosuppressive effect of MSC. Prostaglandin E2 (PGE2), transforming growth factor-β (TGFβ), heme oxygenase-1 (HO1), interleukins-6 and 10 (IL6 and IL10), human leukocyte antigen-G5 (HLA-G5), HGF, matrix metalloproteinases, indoleamine-2, 3-dioxygenase (IDO), and nitric oxide (NO) are all candidates under investigation.14

Many of these soluble factors such as PGE2, TGFβ, HO1, IL6, IL10, HLA-G5, and HGF are constitutively produced by MSC.17-24 The secretion of certain molecules can be further increased when MSC are stimulated. For instance, TNFα and IFNγ have been shown to increase the production of PGE2 by MSC, which in turn mediates the suppressive effects of MSC on TNFα secretion from mature DC, IFNγ secretion from T lymphocytes, and PHA-induced lymphocyte proliferation.18 IL6 has also been reported to be involved in the inhibitory effect of MSC on mixed lymphocyte reaction as well as the differentiation of bone-marrow progenitor cells into dendritic cells (DC).25 Another important molecule HLA-G5 has been shown to regulate activity of MSC to suppress T-cell proliferation, as well as NK-cell and T-cell cytotoxicity, and to promote the generation of regulatory T cells.23 Cell contact between MSC and activated T cells induces IL10 production, which, in turn, has an essential role in stimulating the release of soluble HLA-G5 by MSC.19

On the other hand, certain factors in MSC secretome are only released following crosstalk with target cells. For instance, IDO is only secreted by MSC when stimulated by IFNγ. IDO contributes to the inhibition of lymphocyte proliferation by MSC through depletion of tryptophan, which is an essential amino acid for lymphocyte proliferation. MSC-derived IDO was also required to inhibit the proliferation of IFNγ-producing TH1 cells.26 IFNγ, alone or in combination with TNFα, IL1α or IL1β, also stimulates the production of inducible nitric-oxide synthase (iNOS), which inhibits T-cell activation through the production of NO.27 MSC from mice deficient for the IFNγ receptor IFNγR1 do not have immuno-
suppressive activity, which further support the crucial role of IFNγ-indicible factors to activities of MSC.\textsuperscript{27}

In additional to these molecules, TSG-6 has recently been identified as mediating anti-inflammatory activities of BMSC in several inflammatory disease models. Its effects on different immune cells, such as lymphocytes remain unknown. It is therefore highly interesting to find out if TSG-6 would shed some new light into the sophisticated immunemodulatory effects of MSC.

MSC and Vascular Barrier

Vascular endothelium serves both as a regulator and victim in the inflammatory process. One critical early step of inflammation, leukocyte extravasation, is tightly regulated by adhesion molecules and junction proteins on the surface of endothelial cells.\textsuperscript{28} BMSC has been shown to inhibit VEGF-induced permeability to dextran \textit{in vitro} by increasing VE-cadherin levels and enhancing recruitment of both VE-cadherin and beta-catenin to the plasma membrane of endothelial cells.\textsuperscript{29} In addition, leukocyte adhesion and adhesion molecule expression (VCAM-1 and ICAM-1) were also inhibited in pulmonary endothelial cells (PEC) treated with conditioned media from MSC-PEC co-cultures. By stabilizing vascular endothelium in inflammation, MSC were able to significantly reduce leukocyte infiltration and edema in lung in a rat model of hemorrhagic shock-induced lung injury.\textsuperscript{30} Despite these previous descriptions of protective effects of MSC on endothelial integrity, the key mediators responsible for activities of MSC remain yet to be found.

Given the recently described \textit{in vivo} perivascular location of both ASC and other MSC,\textsuperscript{31, 32} we felt that an important set of experiments, complementary to those above, would address the crosstalk between MSC and EC in the context of inflammation, especially with regard to leukocyte transmigration. These experiments are also described in Chapter 2.
1.2 Tumor Necrosis Factor-Stimulated Gene 6 (TSG-6)

Regulation of TSG-6 Transcription

Tumor necrosis factor alpha-induced protein 6 (TNFIP6 or TSG-6) was first discovered in TNFα-stimulated human foreskin fibroblasts. The coding gene for TSG-6 is located in chromosome 2q23.3. Sequences responsible for activation of TSG-6 transcription are located between positions -165 and -58. Sequencing of a 1.3-kilobase fragment of the 5'-flanking region of the TSG6 gene identified TATA-like and CAAT sequences near the transcription start site. In addition, potential binding sites for interferon regulatory factors (IRF), activator protein-1 (AP1), nuclear factor interleukin-6 (NFIL6), and glucocorticoid response elements (GRE) have been identified in the 5'-flanking region (Figure 2A) and may be involved in TNFα and IL1β-induced TSG-6 activation, although these two cytokines may act through distinct pathways. The region further upstream (between positions -332 and -165), however, may be involved in silencing of TSG-6 transcription.

The inducibility of TSG-6 is regulated differently depending on the cell type. In addition to fibroblasts, Many other types of primary cells were also capable of increasing TSG-6 production under a variety of stimulatory conditions, such as neutrophils (stimulated by lipopolysaccharides), smooth muscle cells (by mechanical strain), and cumulus oocyte complex (by ovulation) (reviewed in 35). Occasionally, certain types of cells have been shown to produce TSG-6 constitutively, such as human amniotic membrane epithelial and stromal cells. Interestingly, cycloheximide (protein synthesis inhibitor) did not interfere with transcriptional activation of TSG-6 by TNFα in fibroblasts, but abrogated growth factor-induced TSG-6 expression in smooth muscle cells. This suggests that pathways regulating TSG-6 expression may vary among different cell types.

More than 400 single nucleotide polymorphisms (SNP) have been noted in human TSG-6 gene. Nentwich et al reported a particular SNP that involves a non-synonymous G to A transition at nucleotide 431, resulting in an Arg to Gln alteration in the CUB module. Although modeling indicated that the amino acid change might lead to functional differences, no association was found between...
the polymorphism and susceptibility to osteoarthritis in the 400 osteoarthritis cases and 400 controls studied.\textsuperscript{38}

Structure and Binding Partners

In fibroblasts, after cleavage of the signal peptide and N-glycosylation, TSG-6 is secreted as a 35 kDa glycoprotein.\textsuperscript{39} As a member of the hyaluronan-binding protein family, TSG-6 protein consists of two structural domains: a N-terminal hyaluronan-binding link module, the characteristic domain of this family of proteins, and a C-terminal CUB domain (Figure 2B). NMR spectroscopy has revealed that the Link module comprises two triple-stranded antiparallel β-sheets and two α-helices arranged around a large, well-defined hydrophobic core. Other proteins sharing the link module with TSG-6 include CD44, aggrecan, and versican. Via the link module, TSG-6 interacts with a broad spectrum of GAG (glycosaminoglycan) and protein ligands, including HA (hyaluronan), C4S (chondroitin-4-sulphate), heparin, IαI (inter-α-inhibitor), CD44, aggrecan, versican, TSP1 (thrombospondin-1), and PTX3 (pentraxin-3).\textsuperscript{39-47} On the other hand, TSG-6 also binds to fibronectin via the CUB module, but not the link module, and mediates fibronectin interactions with other matrix components and cells.\textsuperscript{48}
Figure 2. Gene and protein structure of TSG-6.

(A) The TSG-6 promoter contains potential binding sites for interferon regulatory factors (IRF, at -129, -947, and -1016), activator protein-1 (AP-1, at -126), nuclear factor interleukin-6 (NFIL-6, at -115 and -1291), and glucocorticoid response elements (GRE, at -629 and -1148). (B) TSG-6 protein consists of a link module (Gly^{36} to Cys^{127}) and a CUB module (Gly^{136} to Phe^{240}) with two potential glycosylation sites (ball and stick).
TSG-6 in Inflammatory Diseases

The inducibility of the TSG-6 gene by the proinflammatory cytokine TNFα suggested a possible association with inflammatory processes and a potential role in inflammatory diseases. Indeed, TSG-6 was absent in normal synovial fluids but became readily detectable in synovial fluids of patients presenting with various joint diseases including rheumatoid arthritis, osteoarthritis, Sjogren’s syndrome, polyarthritic gout, and osteomyelitis. This is consistent with the in vitro observation that synovial cells isolated from patients with rheumatoid arthritis expressed TSG-6 constitutively and responded to stimulation with either IL-1β or TNFα with an additional upregulation of TSG-6 expression. Besides synovial fluid, TSG-6 was also found to be high in the sera of patients with bacterial sepsis and systemic lupus erythematosus.

Use of Recombinant TSG-6 Protein in Animal Models of Inflammation

The in vivo anti-inflammatory effect of TSG-6 was recognized more than a decade ago first in a skin inflammation model, in which local injection of 10 µg recombinant human TSG-6 protein significantly attenuated both carrageenan and IL1β-induced edema and neutrophil infiltration in the subcutaneous air pouch, similar in magnitude to that seen with dexamethasone. Bioling or single amino acid substitutions in the N-terminal region resulted in complete loss of TSG-6 activity, suggesting the importance of link module to the activity of TSG-6.

Later in a proteoglycan-induced arthritis model, recombinant murine TSG-6 were administered either intravenously or intra-articularly. Intravenous injection (100 µg) of rmTSG-6 induced a dramatic reduction of edema in acutely inflamed joints by immobilizing CD44-bound hyaluronan and, in long-term treatment, protected cartilage from degradation and blocked subchondral and periosteal bone erosion in inflamed joints. The intra-articular injection of a single dose (100 µg) of rmTSG-6 exhibited a strong chondroprotective effect for up to 5 to 7 days, preventing cartilage proteoglycan from metalloproteinase-induced degradation. However, the onset and incidence of arthritis were not affected, nor were any changes in serum pro- and anti-inflammatory cytokines observed. The
importance of TSG-6 to inflammation control was further strengthened by the observation that TSG-6-null mice suffered from more extensive and rapid cartilage degradation, bone erosion, joint ankylosis, and deformities in a proteoglycan-induced arthritis model.\textsuperscript{53}

TSG-6 and Marrow Stromal Cells

The variety of inflammatory disease models found to be improved by TSG-6 has rapidly increased since TSG-6 was first identified in the secretome of bone marrow stromal cells (BMSC). Lee \textit{et al} found that intravenously delivered BMSC were mostly trapped in the lung and activated by TNFα to secrete TSG-6, in the context of cardiac injury. Knockdown of TSG-6 by siRNA led to loss of the therapeutic effects of BMSC on infarcted myocardium.\textsuperscript{54} Furthermore, similar to previous observations in arthritis model, the protective effects of BMSC and TSG-6 protein on myocardium were also associated with inhibition of plasmin activity and MMP9 activation, and reduction of neutrophil infiltration.

Such overlap between indications of BMSC and TSG-6 has fueled successive efforts to substitute BMSC-based cell therapy with TSG-6-based cytokine therapy. In a corneal injury model, in which BMSC have previously been proven effective, local injection of 2 µg TSG-6 resulted in marked decrease of corneal opacity, neovascularization, and neutrophil infiltration, accompanied by reduced proinflammatory cytokines (IL6 and IL1β), chemokines (CXCL1 and MCP1), and matrix metalloproteinases (MMP9).\textsuperscript{55} Similar protease suppressive activity, not on MMP9 but on MMP1 and MMP3, was responsible for the anti-inflammatory function of TSG-6 produced by conjunctiva fibroblasts in a conjunctivochalasis model.\textsuperscript{56} Local injection of 400 ng TSG-6 also led to slower progression or alleviation of retinal lesions in a murine model of focal retinal regeneration.\textsuperscript{57} In a peritonitis model, intraperitoneous injection of 30 µg recombinant human TSG-6 exhibited equivalent effect as 1.6 X 10^6 human BMSC in reducing total cell numbers in the exudate.\textsuperscript{47} In addition, the anti-inflammatory activity of TSG-6 comparable to BMSC was also demonstrated in an acute lung injury model.\textsuperscript{58}
Modulation of Immune Cells by TSG-6

As a member of the hyaluronan-binding protein family, TSG-6 consists of “link” and “CUB” modules. The shared structure of link modules in this protein family allows for binding of the TSG-6 link domain to other members such as hyaluronan, chondroitin-4-sulfate, proteoglycan aggrecan, and CD44. Among them, CD44 was found to be a receptor for TSG-6 on macrophages mediating a feedback inhibition on TNFα release via interference with the NF-κB signaling pathway. Cao et al observed that neutrophil adhesion and extravasation in microcirculation were significantly reduced by the administration of link module of TSG-6 protein; and similarly, transmigration of neutrophils across endothelial cell monolayers in vitro was also decreased by the link module as well as the TSG-6 holoprotein. Unfortunately, they were not able to identify any specific responsible molecular mechanisms since the three measured adhesion molecules (ICAM-1, PECAM-1, and P-selectin) of endothelial cells remain unchanged.

Protease and Extracellular Regulation by TSG-6

Protease network regulation is another proposed mechanism of TSG-6. According to Wisniewski et al, TSG-6 can form a complex with inter-α-inhibitor, which exhibits potent anti-plasmin activity. This could explain the attenuated tissue damage after TSG-6 injection, since the two major destructive proteases released from neutrophils, matrix metalloproteinase 2 and 9 (MMP2 and MMP9), are activated by plasmin. On the other hand, the protective effect of TSG-6 may not be restricted to reducing inflammatory attacks but also enhancing the protective layer of extracellular matrix. Finally, the link module of TSG-6 has a strong affinity for hyaluronan, and hyaluronan-crosslinking via TSG-6 at the surface of synovium, cartilage, or oocyte may form a protective barrier, potentially preventing matrix degradation, and/or acting as a scaffold for matrix regeneration.

While previous studies of TSG-6 have primarily focused on proteases and extracellular matrix as its targets, we felt that it would be novel and potentially important to evaluate its effects on immune cells and how it contributes to the
anti-inflammatory effects or immunomodulation by MSC. This became an important motivation for several of the experiments described in Chapter 2.

1.3 Cigarette Smoking-induce Myelotoxicity as an Inflammatory Disease Model

Clinical Evidence for Extrapulmonary Toxicity of Smoking

CS is one of the most prevalent life style risk factors adversely influencing the health of human beings. On average, one in two men and one in ten women are current smokers. It has been increasingly recognized that the damage from cigarette smoking is not only restricted to the lung but also extends to extrapulmonary organs, including well-established links to atherosclerosis, peripheral arterial disease, and cancer.\textsuperscript{60-64}

Compared to other organs, the toxicity in bone marrow caused by CS tends to be more insidious, with little or no clinical manifestations. This is probably because only 10-20\% of the normal complement of hematopoietic progenitors is necessary to maintain hematopoiesis, and bone marrow is not routinely examined in otherwise healthy smokers.\textsuperscript{65, 66} Nevertheless, there has been accumulating evidence showing that CS is associated with an increased risk of bone marrow failure, myelodysplasia, and myeloid leukemia, as well as up to 50\% reduction in survival following marrow transplantation into smokers.\textsuperscript{67-69}

Myelotoxicity in Animal Models

In animal models, Khaldooyanidi \textit{et al} observed that after 2 weeks of CS exposure, bone marrow myeloid progenitors in BALB/c mice decreased by 53\% and the homing of hematopoietic progenitors to bone marrow after lethal irradiation decreased by 83\%. This myelotoxicity of CS was consistent with the \textit{in vitro} finding that formation of cobblestone areas was inhibited by nicotine treatment in long-term bone marrow culture and that colony formation from hematopoietic progenitors was inhibited by a metabolite of nicotine, cotinine.\textsuperscript{70, 71} Although nicotine does promote extra-medullary hematopoiesis, subcutaneously implanted nicotine, after 3 weeks of slow release, was not able to fully
recapitulate the myelosuppressive effect of smoking on the bone marrow. These findings underline the complexity of chemical compounds in smoke other than nicotine, which also play important roles in the pathogenesis of smoking toxicity.

Mediators for Systemic Toxicity of Smoking

Other critical factors to be considered are derivatives from body response to smoking, such as acute-phase proteins (APP), inflammatory cytokines and free radicals. Several studies have reported strong associations between cigarette smoking and different APP such as C-reactive protein (CRP) and fibrinogen. CRP might contribute to the increased risk of atherosclerosis and endothelial dysfunction in smokers, by stimulation of IL6 and endothelin-1 production, and upregulation of adhesion molecules, promoting a cascade of events that can lead to clot formation and even atherosclerosis in apolipoprotein E-deficient mice. Fibrinogen may promote cardiovascular diseases through effects on blood viscosity, platelet aggregation, and fibrin formation. On the other hand, raised levels of plasma APP may also reflect elevations of inflammatory cytokines such as IL6 and TNFα, which are major inducers of APP. In fact, several studies have indeed shown increased levels of TNFα and IL6 in smokers. In addition, Exposure to oxidant chemicals in smoke is associated with depletion of endogenous levels of antioxidants in the systemic compartment. The total plasma antioxidant capacity was significantly lower in smokers than in nonsmokers and an inverse relationship between cigarette consumption and plasma levels of vitamin C and β-carotene corrected for habitual dietary intake has been found. These byproducts from smoking may be just as detrimental as the original chemical compounds contained in the cigarette smoke. For instance, both CRP and TNFα are established suppressors of colony formation from hematopoietic progenitors.
1.4 Mesenchymal Stem Cells and Hematopoiesis

BMSC & Hematopoiesis

In bone marrow, various chemokines, extracellular matrix and adhesion molecules produced by stromal cells contribute to the formation of “stem cell niches”, which regulate homing, proliferation, differentiation, and mobilization of hematopoietic progenitor cells\textsuperscript{84-90}. More specifically, MSC produce stem cell factor, Fms-like Tyrosine Kinase (FLT3) ligand, thrombopoietin, leukemia-inhibiting factor, TGF\textbeta, IL4, IL6, IL7, IL8, IL11, IL12, IL15, granulocyte macrophage (GM-) and macrophage colony stimulating factor (M-CSF).\textsuperscript{91} MSC also synthesize intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), as well as laminin, fibronectin, collagen and proteoglycans.\textsuperscript{92, 93} This niche structure built from MSC even without other cell types was sufficient to maintain long-term culture initiating cells and expand lineage-specific colony forming units from CD34\textsuperscript{+} bone marrow cells in long-term bone marrow cultures.\textsuperscript{94}

Hematological Therapy involving BMSC

Normal hematopoiesis in the bone marrow is subject to many environmental or interventional hazards, such toxins, irradiation, and chemotherapy. Refractory myelosuppression warrants bone marrow transplantation, often allogeneic, which is hampered by the severe complication of graft versus host disease (GVHD). The hematopoiesis-supporting and immunomodulatory nature of MSC makes them an ideal adjunctive therapy to prevent / mitigate GVHD and enhance engraftment and fast recovery of hematopoietic stem / progenitor cells. Numerous clinical trials have been conducted testing the efficacy of MSC against de novo or steroid-refractory GVHD. There are still considerable controversies over the outcomes, which seem to be affected by the patient demographics, affected organs, cell infusion scheme, etc. Nevertheless, the potential for MSC clinical benefit appears high. In one study, MSC were given 50-294 days post-transplant without co-infusion of hematopoietic stem cells (HSC), to functionally improve bone marrow
microenvironment and to further stimulate residual hematopoietic tissue. Two out of six patients showed rapid hematopoietic recovery, in contrast to the other heavily pretreated patients.\textsuperscript{95} Interestingly, in a preclinical study, human MSC co-transplanted with human cord blood CD34\textsuperscript{+} cells in irradiated NOD/SCID mice were shown to promote hematological recovery despite an absence of persistent MSC in the bone marrow at 6 weeks post transplantation.\textsuperscript{96} This suggested that infused MSC could promote homing and/or proliferation of HSC via their constitutive secretion of various hematopoietic cytokines and immunomodulatory soluble factors, without chronic persistence of MSC within the recipient bone marrow.\textsuperscript{97}

ASC & Hematopoiesis

In contrast to their counterpart in the BM, ASC have been much less explored in the context of hematopoiesis, possibly due to their long geographic distance from bone marrow \textit{in vivo}. The recent discovery of hemangioblast in the adult adipose tissue sparked the idea that even outside the bone marrow, ASC may still retain important functions involved in the support of hematopoiesis.\textsuperscript{98} Indeed, many of the critical hematopoietic cytokines from BMSC can also be secreted by ASC. Interestingly, Nakao and De Toni in independent studies both found that ASC demonstrated an even stronger benefit than BMSC in facilitating engraftment of HSC after mice were irradiated.\textsuperscript{99, 100} Little has been known regarding to whether and how ASC protect hematopoietic stem and progenitor cells from myelotoxins such as free radicals, inflammatory cytokines, and chemotherapy agents. These findings, taken together, provided the inspiration for the experiments concerning the effects of ASC and TSG-6 on CS-induced myelotoxicity, described in Chapter 4.
Chapter 2. Inflammation Control through TSG-6: Dynamics at the Perivascular Niche

2.1 Introduction

The vascular wall formed by endothelial cells (EC) restricts passage of circulating molecules and inflammatory cells into the underlying tissues. This barrier function is critical for the maintenance of tissue homeostasis and cellular functions. ASC reside in perivascular niche in vivo resembling pericytes and, when isolated and administered back, exert potent effects against inflammation.\textsuperscript{31, 32} This anti-inflammatory function of ASC has previously been attributed mainly to the immune-modulation of various immune cells through either direct contact inhibition or soluble factors.\textsuperscript{14} The role of ASC in the regulation of vascular barrier function has rarely been explored.

On the other hand, the distinct contrast between quiescence of ASC in the perivascular niche and strong potency after isolation calls for attentions to the niche suppression of ASC behavior, especially by neighboring EC. Indeed, it has been noted that adipogenic differentiation of ASC is inhibited by EC so that more ASC can be preserved to stabilize vasculature.\textsuperscript{101} It remains unknown how EC affects anti-inflammatory functions of ASC.

Tumor necrosis factor-induced glycoprotein 6 (TSG-6) was first discovered in TNFα-treated fibroblasts.\textsuperscript{33} TSG-6 expression has been observed in physiological and pathological contexts associated with inflammation and tissue remodeling, for example in the synovial fluid of arthritis patients and serum of lupus patients.\textsuperscript{49, 50} Local or systemic injection of TSG-6 has been proven to effectively attenuate inflammation in a diversity of disease models, including arthritis, myocardial infarction, corneal injury, acute lung injury, and peritonitis, to name a few.\textsuperscript{47, 54, 55, 58} Histology of affected tissue in these models consistently showed significantly reduced infiltration of leukocytes as a result of TSG-6 injection. Reported mechanisms of TSG-6 include: 1) it inhibits the inflammatory network of proteases primarily by increasing the inhibitory activity of inter-a-inhibitor; 2) it binds to fragments of hyaluronan and thereby blunts their proinflammatory effects; 3) it inhibits TNFα secretion from macrophages. How
TSG-6 affects endothelial permeability and lymphocyte proliferation, two critical steps in the inflammatory cascade, remains unknown.

We aimed to address these questions by constructing in vitro models to study interactions between ASC and vascular EC in the context of inflammation. We also looked into the effect of TSG-6, a critical anti-inflammatory cytokine that has previously not been noticed in the secretome of ASC, in these models to better understand its mechanism of mitigating inflammation.

2.2 Materials and Methods

Cell culture

Human ASC were isolated from human subcutaneous adipose tissue samples obtained from liposuction procedures, as previously described. Briefly, samples were digested in collagenase Type I solution (Worthington Biochemical) under agitation for 1 hr at 37°C, and centrifuged at 300 g for 8 min to separate the stromal cell fraction (pellet) from adipocytes. The pellets were filtered through 250 µm Nitex filters (Sefar America Inc.) and treated with red cell lysis buffer (154 mM NH₄Cl, 10 mM KHCO₃, and 0.1 mM ethylenediamine-tetraacetic acid). The final pellet was resuspended and cultured in EGM-2MV growth media (Lonza). ASC were passaged when 60–80% confluent and used at passages three to five.

Human umbilical vein endothelial cells (HUVEC) were purchased from Lonza and cultured in EGM-2 media per manufacturer’s instruction. Passage 8-10 HUVEC were used for this study. For co-culture, ASC at 6 X 10⁴ cells/cm² and HUVEC at 5-10 X 10³ cells/cm² were premixed before plating and then cultured in EBM-2/5% fetal bovine serum (FBS) for 6-8 days with medium change every 3 days. To compare TSG-6 secretion profile, ASC cultured alone or co-cultured with EC were washed with phosphate buffer saline (PBS, Gibco) on day 7 and treated with 20 ng/ml human TNFα (R&D systems) in fresh EBM-2 medium. After 24 hrs, culture supernatant was collected and concentrated by 8-10 fold using 10 kD Amicon Ultra centrifugal filter (Millipore) for analysis of TSG-6 levels.
Western Blot for human TSG-6

To measure the secretion of human TSG-6 protein, Western blot analysis was performed. Concentrated culture media samples were separated by electrophoresis on 10% Precise Protein Gels (Thermo Scientific) under denaturing and reducing conditions. Proteins in gels were transferred to a nitrocellulose membrane, which was then blocked with 5% (wt./vol.) fat-free milk in PBST (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% [vol./vol.] Tween-20), followed by sequential incubation with human TSG-6 affinity purified goat IgG (R&D systems, 1:200), and Donkey anti-goat secondary antibody (Santa Cruz, 1:5000), using nonspecific Naphthol blue black (Sigma) staining as the loading control. Immunoreactive proteins were detected with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific).

PMN and Lymphocyte Isolation

Polymorphonuclear cells (PMN) and lymphocytes were isolated from peripheral blood using Ficoll-Paque Plus (GE Healthcare Life Sciences) according to manufacturer’s instructions. Briefly, 30 ml peripheral venous blood from healthy human donors was centrifuged at 900 g for 10 min. The plasma was removed, and the leukocyte-rich upper layer was transferred to a new tube and diluted to 30 ml with Hank’s Balanced Salt Solution (HBSS, Thermo Scientific). The cell suspension was then layered onto 15 ml Ficoll-Paque Plus (GE Healthcare Life Sciences). After centrifugation at 400 g for 40 min, the sample was divided into layers of (from top to bottom) plasma, lymphocytes, Ficoll-Paque Plus, PMN, and erythrocytes. Lymphocytes and PMN were collected from corresponding layers, incubated in erythrocyte lysis buffer for 10 min and washed twice with HBSS before immediate use.

FITC-bovine serum albumin (BSA) Permeability Assay

We performed permeability assays across HUVEC monolayers using a Transwell system (Figure 3) as previously described. Transwell permeable supports (Corning) with polycarbonate membranes (6.5 mm in diameter, 3 µm
pore size and pore density of $10^8$/cm$^2$) were coated with Matrigel (BD Biosciences) for 1 hr at 37°C. The membranes were seeded with HUVEC growing in EGM-2 media until they formed a complete monolayer. The integrity of confluent EC monolayer was assessed by Alexa Fluor® 488 Phalloidin (Invitrogen) staining and by including a non-treated control in each experiment. To induce permeability, human thrombin (10-50 U/ml, Sigma) together with 100 µg/ml FITC-BSA (Sigma) was added to the lower compartment. At different time points after treatment, 10 µl of media from the upper compartment were sampled and diluted in 90 µl water/well in a 96-well plate. Fluorescent intensity was measured on a fluorometer (SpectraMax M5; Molecular Devices) with excitation at 485 nm and emission at 535 nm.

PMN Transmigration through HUVEC Monolayer
Transwell inserts were coated with HUVEC monolayer in the same way as for the FITC-BSA permeability assays. For thrombin-induced transmigration assay, EC monolayer was serum-starved for 2 hrs before adding $2 \times 10^6$ PMN in the volume of 100 µl to the upper chamber and thrombin (10 U/ml, Sigma), D-Phenylalanyl-L-Prolyl-L-Arginine Chloromethyl Ketone (PPACK, thrombin inhibitor, 0.1 µM, Millipore), or TSG-6 (100 ng/ml, R&D systems) were added to the lower compartment. For ASC treatment groups, the lower compartment has been coated with confluent human ASC one day before the experiment. After 4-6 hrs, cells that had migrated into the lower compartment were collected, stained with FITC-CD11b Ab (BD Biosciences), and counted on flow cytometer (Guava EasyCyte 8HT, Millipore).

For TNFα-induced transmigration assay, PMN were labeled green using CellTrace™ CFSE (Invitrogen) before loading to the upper chamber. After serum starvation of EC monolayer for 3 hrs and incubation with human TNFα (20 ng/ml) for 3 hrs, inserts were removed and cells migrated into the lower compartment were counted under fluorescent microscope (Eclipse Ti, Nikon).
Figure 3. Polymorphonuclear cell (PMN) transmigration assay.
A lateral view of the Transwell system is shown. Human umbilical vein endothelial cells (HUVEC) were seeded until confluent in Matrigel-coated transwell inserts (3 µm pore size and a surface area of 0.33 cm²). Human adipose stem cells (hASC) were seeded in the lower compartment without inserts on top at first. HUVEC monolayer was serum-starved for 2 hrs and incubated with PPACK (thrombin inhibitor), TSG-6, or hASC for 30 min before treatment of thrombin or TNFα. There was no physical contact between HUVEC and hASC. Permeability of endothelial monolayer to PMN was assessed by adding PMN only to the upper compartment and measuring their unidirectional flux to the lower compartment. There was no hydrostatic pressure gradient between compartments.
Lymphocyte Proliferation Assay

The mononuclear cell (MNC) fraction was stimulated with mouse anti-human CD3 (1 µg/ml; BD Biosciences) and mouse anti-human CD28 (0.5 µg/ml; BD Biosciences). Cell cultures were then incubated for 6 days at 37°C / 5% CO₂. Eighteen hours before the end of the experiment, ³H-thymidine (0-5 mCi/well) was added to each of the wells. On day 7, cell proliferation was determined by measuring the incorporation of ³H-thymidine using a β-plate reader and quantified as counts per minute (cpm).

Statistical Analyses

Data are shown as mean ± standard error (SEM). Comparisons between two groups were made with the use of analysis of variance (ANOVA) and Newman-Keuls post-hoc analysis. A p value of < 0.05 was considered significant.
2.3 Results

Niche Endothelial Cells Inhibit Secretion of TSG-6 from ASC

Previous studies have shown that ASC reside in perivascular niche, in the neighborhood of endothelial cells (EC).\textsuperscript{32} To simulate their \textit{in vivo} niche environment, we cultured ASC with EC for 7 days. EC (positively stained with FITC-CD31) distributed randomly on plates in early days (day 2) but gradually anastomosed with each other and aggregated to form cord-like structures, much resembling their behavior as vascular blocks during neovascularization (Figure 4A). This was accompanied by migration of ASC to surround these cord-like structures. Interestingly, in this peri-endothelial cell niche, the secretion of TSG-6 from ASC induced by TNFα was significantly suppressed compared to that from ASC cultured alone (Figure 4B). The suppressive effect of EC on ASC suggests that EC play an important regulatory role in the quiescence of ASC located in peri-vascular niche; and that prominent therapeutic effects observed in isolated ASC may be partly due to enhanced secretions of TSG-6 after ASC were liberated from suppression by EC.
Figure 4. Niche endothelial cells suppress TSG-6 secretion from ASC.
(A) Cord-like structures were formed by migrated endothelial cells and surrounded by ASC after co-culture for 8 days. (B) Neighboring endothelial cells (HUVEC), which do not produce TSG-6 by themselves, suppressed TNFα-induced secretion of TSG-6 from ASC. Blots are representative of 3 independent experiments.
Effects of ASC and TSG-6 on thrombin-induced hyperpermeability to Albumin

To determine the reciprocal effect of ASC on EC and the role of TSG-6, we next looked into effects of ASC and TSG6 on endothelial barrier function. Vascular leakage to proteins such as albumin is the major cause of edema in inflammation. We used an EC monolayer on Matrigel-coated transwell inserts to mimic the intact vascular wall barrier. We also used thrombin, which is activated from prothrombin on the surface of endothelial cells when inflammation occurs, as an inducer of hyperpermeability as described previously.\textsuperscript{104} We chose the dose of 10 U/ml for all experiments in this study because a lower dose tested (1 U/ml) was not sufficient to induce leaking of EC monolayer (Figure 5A). Treatment with 10 U/ml human thrombin resulted in an acute progressive leaking of FITC-BSA across the endothelial monolayer, which cannot be further augmented with higher dose (Figure 5B). The permeability to albumin induced by thrombin was decreased by thrombin inhibitor PPACK and equally by human ASC without contact with EC, but not by 100 ng/ml (higher than baseline secretion level in ASC supernatant) TSG-6. This protective effect became more prominent after prolonged treatment (from 4 to 72 hrs) of thrombin (Figure 5B). These results indicate that TSG-6 alone could not substitute the inhibitory effect of ASC on thrombin-induced leaking of albumin; and there may be other factors in the ASC secretome that contribute to the protection of endothelial barrier.
Figure 5. Effects of ASC and TSG-6 on permeability of EC monolayer to FITC-bovine serum albumin (BSA).

Permeability across EC monolayer was determined by measuring intensity of fluorescence (relative fluorescent unit) in the upper compartment (FITC-BSA was added to the lower compartment). (A) Permeability to FITC-BSA began to increase, compared to non-treated control, approximately 1 hr after thrombin treatment. Strongest induction was achieved with 10 U/ml thrombin. (B) ASC significantly reduced thrombin-induced EC permeability to the level of non-treated control and thrombin inhibitor (PPACK) groups. Inhibitory effects of PPACK and ASC on EC permeability became more obvious with prolonged treatment time (24 hrs and 72hrs). Such effect was not observed in the TSG-6
group. Data shown are mean ± SEM in one representative experiment. 

\(^a\) p<0.05 (vs. non-treated control, NC); 

\(^b\) p<0.05 (vs. vehicle).
Effects of ASC and TSG-6 on thrombin-induced PMN Transmigration

Compared to leaking of albumin, extravasation of neutrophils across endothelium is governed by distinct mechanisms involving adhesion molecules and junctional proteins on endothelial cells. Thrombin induces leukocyte extravasation by 1) enhancing chemotaxis of leukocyte; 2) promoting adhesion of leukocyte to endothelium; and 3) dissociating intercellular junctions between endothelial cells.\textsuperscript{104} We observed minimum transmigration of PMN in the non-treated group, which confirmed the integrity of a confluent EC monolayer. Treatment with 10 U/ml human thrombin elicited vast PMN transmigration across the monolayer, which was abrogated when thrombin was neutralized by its inhibitor PPACK. Co-cultured hASC in the lower compartment, without physical contact with EC, also reduced the migration of PMN, to the same effect as PPACK. TSG-6 protein (100 ng/ml) did not stop the leaking of FITC-BSA, but it did inhibit PMN transmigration across endothelial monolayer, although the effect was weaker than that of ASC (Figure 6).
Figure 6. Thrombin-induced PMN transmigration across EC monolayer and modulatory effects of ASC and TSG-6.

Migrated PMN were floating in the lower compartment (white dots) while human ASC (ASC group) were attached to the bottom (spindle shaped). Integrity of EC monolayer was confirmed by minimum migration of PMN to the lower compartment (non-treated group) and absence of intercellular gaps upon Alexa Fluor® 488 phalloidin staining of actin (green).
To quantify migrated PMN in the lower compartment without interference from co-existing hASC, all cells in the lower compartment were trypsinized, collected, stained with CD11b Ab, and counted on flow cytometry. The accuracy of this method is justified by the linear standard curve showing positive correlation between events on flow cytometry and the number of PMN (counted by hemocytometer) in each sample (Figure 7). In accordance with microscopic findings, PPACK, hASC and TSG-6 reduced the transmigration rate of PMN from 3.8 ± 0.6% to 0.2 ± 0.02%, 0.1 ± 0.02% and 2.0 ± 0.5%, respectively (Figure 8B). Since ASC were not in direct contact with EC on inserts, the potent inhibitory effect of ASC was most likely attributed to cell-secreted trophic factors, such as TSG-6. This speculation motivated our further efforts to look into the efficacy of ASC conditioned media.
Figure 7. Quantification of thrombin-induced PMN trans-endothelial migration by flow cytometry.

(A) Flow cytometry allows for distinction between migrated PMN and ASC in the lower compartment. (B) Live PMN (Calcein AM⁺ CD11b⁺) were counted for each sample. (C) PMN stained with IgG isotype Ab were CalceinAM⁺ CD11b⁻. (D) PMN stained with CD11b Ab but killed by methanol were CalceinAM⁻ CD11b⁺. (E) The flow cytometry-based counting of viable PMN is proportional to the number of PMN (counted by hemocytometer) in each sample.
Effects of ASC conditioned media on thrombin-induced PMN Transmigration

If ASC do function through certain trophic factors, such as TSG-6, conditioned media from ASC should yield equivalent effects. The challenge is that it is difficult to calibrate the condition media to precisely recapitulate activities of ASC because ASC might be affected remotely by the presence of other cell populations in the model, such as EC and PMN or other stimulatory signals such as thrombin. To take into account possible influences from these environmental factors, we generated conditioned media from several different culture conditions, including 1) hASC only; 2) hASC (in lower compartment) + EC (on inserts); 3) hASC + EC + PMN (in upper compartment); 4) hASC + thrombin; 5) hASC + EC + thrombin; 6) hASC + EC + PMN + thrombin (Figure 8A). These conditioned media were used to replace assay media for the next PMN transmigration assay for efficacy evaluation. Surprisingly, thrombin in conditioned media from ASC alone or ASC + EC induced higher permeability than in regular assay media, whereas thrombin in conditioned media from ASC + EC + PMN induced the same level of permeability as in regular assay media (Figure 8C). Moreover, adding thrombin to the conditioned media generation process also failed to induce any anti-permeability effect of the conditioned media (Figure 8D). These results showed that, at least in this assay, the presence of ASC is indispensible to maintain their barrier-protective effect.
Figure 8. Effects of ASC, ASC conditioned media, and TSG-6 on thrombin-induced PMN transmigration.

(A) Conditioned media was harvested from ASC, ASC + EC, or ASC + EC + PMN, incubated with (CMAT, CMAET, CMAEPT, respectively) or without (CMA, CMAE, CMAEP, respectively) 10 U/ml thrombin for 6 hrs and frozen until use. (B) ASC completely abrogated thrombin-induced PMN transmigration, comparable to PPACK (PPK). TSG-6 significantly reduced the permeability, but less effectively than ASC. (C) Conditioned media from ASC (CMA) or ASC + EC culture (CMAE) in the absence of thrombin increased PMN transmigration, whereas conditioned media from ASC + EC + PMN (CMAEP) had no effect. (D) Conditioned media from thrombin-stimulated ASC (CMAT), ASC + EC (CMAET), or ASC + EC + PMN (CMAEPT) did not show any inhibitory effect either. Mean ± SEM, n=3/group. a*p<0.05, (vs. non-treated control, NC); b*p<0.05, vs. (vehicle).
Effects of ASC and TSG-6 on TNFα-induced PMN Transmigration

The pro-inflammatory cytokine TNFα is another potent inducer of vascular permeability during inflammation. In contrast to thrombin, TNFα triggered endothelial permeability mainly through microfilament rearrangement, microtubule destabilization, and disruption of vascular endothelial (VE)-cadherin-based intercellular junctions. Therefore, unlike acute-acting thrombin, TNFα increases endothelial permeability progressively within the first 8-10 hrs and maintains its effect for more than 24 hrs. To investigate effects of hASC and TSG-6 against TNFα-induced hyperpermeability, we employed a similar transwell model as the one for thrombin, except this time, we pre-labeled PMN with fluorescent tracker carboxy fluorescein diacetate succinimidyl ester (CFSE) so that they can be counted distinctly from nonfluorescent ASC under fluorescent microscope. Similar to our findings in thrombin-induced endothelial permeability, both hASC and TSG-6 significantly attenuated PMN transmigration from 4.0 ± 0.5% to 1.2 ± 0.3% and 0.9 ± 0.3% respectively (Figure 9). The high similarities between effects of ASC and TSG-6 in this assay further support a potential role of TSG-6 in mediating anti-permeability actions of ASC. To prove this hypothesis, it is necessary to knock down TSG-6 and evaluate its influence on anti-inflammatory activities of ASC, this is addressed in Chapter 3.
Figure 9. Transmigration of PMN induced by TNFα and inhibitory effects of hASC and TSG-6.

Human PMN were labeled with CFSE cell tracker (green) before adding to the upper compartment. Images were taken from lower compartment after TNFα treatment for 3 hrs. Mean ± SEM, n=3/group. \(^a p<0.05\) (vs. NC); \(^b p<0.05\), (vs. vehicle). Images are representative for each condition run in triplicates.
ASC, ASC-CM and TSG-6 Suppress Lymphocyte Activation

The initial tissue damage from neutrophil infiltration is exponentially magnified after cytotoxic lymphocytes are activated into proliferation phase.\textsuperscript{110} To investigate the effect of ASC/ASC-CM/TSG-6 on this phase of inflammation, we incubated human peripheral blood mononuclear cell (MNC) with anti-CD3 and CD28 mAbs, which are known mitogens to stimulate lymphocyte proliferation.\textsuperscript{111} Compared to the non-treated control (0.66 ± 0.07 X 10\textsuperscript{3} cpm), stimulation with anti-CD3 and CD28 mAbs induced strong proliferative responses of lymphocytes (16.9 ± 2.0 X 10\textsuperscript{3} cpm). Co-incubation with human ASC significantly inhibited MNC proliferation, in a dose-dependent manner. This inhibitory effect of hASC can be fully reproduced by conditioned media from hASC activated with 20 ng/ml hTNF\textalpha. Intriguingly, despite all other factors secreted from hASC, TSG-6 alone, also effectively suppressed lymphocyte activation, suggesting an important role of this cytokine in the modulation of lymphocyte proliferation by ASC (Figure 10). Therefore, at least in this model, ASC suppressed CD3/CD28 Ab-activated lymphocyte proliferation via trophic factors, such as TSG-6.
Figure 10. ASC, ASC conditioned media, and TSG-6 attenuate lymphocyte proliferation. Mononuclear cells (MNC) activated by anti-CD3 and anti-CD28 show a significant increase in proliferation compared with inactivated MNC and was attenuated by ASC, ASC-conditioned media (ASC-CM) or TSG-6 protein dose-dependently, as measured by thymidine incorporation. Mean ± SEM, n=4/group. \(^a p<0.05\) (vs. non-treated control); \(^b p<0.05\) (vs. vehicle).
2.4 Conclusion

Our results showed that ASC actively respond to the inflammatory signaling of TNFα by strong upregulation and release of the anti-inflammatory cytokine TSG-6. This feedback response of ASC was suppressed by neighboring niche endothelial cells. We also demonstrated protective effects of ASC on thrombin or TNFα induced hyperpermeability of EC monolayer to albumin or neutrophils. Moreover, the inducible trophic factor TSG-6 from ASC was able to reproduce the inhibitory effect of ASC on thrombin or TNFα-induced neutrophil transmigration across EC monolayer but not albumin leaking. TSG-6 as an anti-inflammatory cytokine newly identified in the ASC secretome was also able to dose-dependently abrogate mitogen-induced proliferation of lymphocytes, similar to ASC or ASC-conditioned media. These findings highlight the orchestrated immunomodulation mediated by TSG-6 when released from ASC upon TNFα activation.
2.5 Discussion

To study the permeability of endothelium, we tried to model the *in vivo* situation by using primary human endothelial cells and transwell filter units coated with Matrigel, extracellular matrix that mimics basal lamina. In addition to commonly used FITC-BSA, we also used migration of neutrophils freshly isolated from human peripheral blood as an indicator for the permeability, because neutrophil infiltration is a hallmark of early-stage inflammation and has been consistently shown to improve after MSC/TSG-6 injection in multiple disease models. The challenge with this approach, however, is the complexity of result interpretation, since smaller number of migrated neutrophils in the lower compartment could be attributed to blocking of inducers (thrombin or TNFα in this case), protection of intercellular junctions and cytoskeleton of endothelial cells, immobilization of neutrophils, or all in combination. Our data showed that human ASC not only reduced leaking of FITC-BSA across EC monolayer but also inhibited leukocyte transmigration induced both by thrombin and TNFα. Such general effects of hASC highly suggest that endothelial cell junction and cytoskeleton, the common ground among these different models, as most likely targets of action. Therefore, next-step efforts for addressing intracellular mechanism should be focused on junction proteins (i.e. VE-Cadherin), endothelial cytoskeletons (i.e. F-actin), as well as their regulatory signaling molecules.

One recent study reported that HUVEC, once cultured with bone marrow stem cells (then separated by sorting), demonstrated higher resistance to vascular endothelial growth factor A (VEGFA)-induced permeability to dextran than those cultured alone.\(^{29}\) In contrast, our model put EC and ASC together in the same reaction system without physical contact to allow for continuous dynamic crosstalk between these two cell types. Intriguingly, in previous report, conditioned media from MSC and EC co-culture but not MSC alone were able to reproduce the protective effect of MSC preconditioning on EC. Although our results also support the hypothesis that the inhibitory effect of ASC derived from paracrine factors not present / active in regular ASC culture media, we did not
observe any effect of conditioned media from various cell combinations either. One possibility could be that these factors were unstable or consumed so quickly that presence of producer cells was indispensible to maintain lasting effects.

Although reduced neutrophil infiltration subsequent to TSG-6 injection was reported in many in vivo studies, the cellular and molecular mechanisms remain a mystery. Our data showed a strong antagonistic effect of TSG-6 against TNFα-induced endothelial hyperpermeability leading to neutrophil extravasation. This finding, combined with the fact that TNFα activates the release of TSG-6 from hASC, revealed a potential feedback protection mechanism mediated by TSG-6 in the perivascular niche triggered by inflammatory cytokine TNFα. CD44, a receptor for TSG-6, is expressed abundantly in endothelial cells and is the key mediator for the protection of hyaluronan and HGF against vascular leakiness.\textsuperscript{112, 113} It has been reported to be upregulated by TNFα in endothelial cells to facilitate monocyte adhesion and transmigration.\textsuperscript{114} Given these connections, it would be interesting to look into changes of CD44 expression and distribution in EC monolayer and determine its relevance to the activities of TSG-6.

The suppression of TSG-6 secretion from ASC by niche EC is interesting but not surprising. After all, there is no need to activate quiescent ASC from the niche when vascular wall is intact in the absence of inflammation. However, when vascular injury occurs and restraints from EC on ASC are removed, secretion of TSG-6 from ASC in response to TNFα serves as a need-based protection mechanism to attenuate inflammation by repairing vascular leakiness and suppressing lymphocyte activation (Figure 11). In case of severe injury when this internal defense mechanism becomes insufficient, harvesting stromal vascular fraction to enrich ASC and delivering them into circulation may be a necessary therapeutic measure, because it would augment the efficiency of TSG-6 production by directly exposing large amounts of ASC to stimulatory signals. Notably, based on our results, this procedure may not be simplified into conditioned media or cytokine cocktails in certain occasions, because activities of ASC may rely on the continuous crosstalk between ASC and nearby cells and environmental signals.
In this chapter we identified TSG-6 in the secretome of ASC and demonstrated its potent anti-inflammatory functions via modulation of endothelial barrier and lymphocyte proliferation, similarly to ASC. However, the relative contribution of TSG-6 to the anti-inflammatory activities of ASC will be elucidated in experiments that investigate ASC effects while TSG-6 secretion is inhibited, described in the next chapter.
Figure 11. Illustration of cell dynamics in perivascular niche in response to inflammation.

1) ASC quiescently reside in perivascular niche in normal vasculature but can be enriched through liposuction and isolation, and administered systemically as a therapeutic measure; 2) when vascular injury occurs, TNFα is released from immune cells and thrombin is activated from prothrombin, both of which cause leaking of vascular wall to albumin and neutrophils (PMN), resulting in edema and tissue damage; 3) tissue injury was worsened in later stage when mitogens induce excessive proliferation of cytotoxic lymphocytes; 4) once ASC are liberated from niche environment, either through vascular injury or more effectively and abundantly through cell isolation, and administered systemically, they respond to TNFα stimulation by highly increased secretion of TSG-6; 5)
TSG-6 not only inhibits extravasation of neutrophils but also suppresses mitogen-induced lymphocyte proliferation.
Chapter 3. Quantitation of TSG-6 Secretion from ASC and Investigation of TSG-6 Role in Rescuing Hindlimb Ischemia

3.1 Introduction

To be able to confidently confirm the knockdown of TSG-6 in ASC, it is essential to accurately quantitate the amount of TSG-6 secretion. Although we were able to detect cytokine TSG-6 in the culture medium by Western blotting, this method became inconvenient to measure large numbers of small-volume samples because it requires concentration of supernatant and depletion of confounding serum proteins. Besides, for cell culture supernatants, it is difficult to find an optimal loading-control protein since products of regular housekeeping genes, such as GAPDH and β-actin, are not secreted into supernatant. In order to better characterize TSG-6 cytokine in different cell types and body fluid specimens, as well as quality control the knockdown of TSG-6 more effectively, an effective method of quantification, such as enzyme-linked immunosorbent assay (ELISA) for TSG-6 is highly warranted. In 2009, Lee et al published the first ELISA protocol for human TSG-6 using antibodies from R&D systems. We optimized this ELISA protocol and used it to verify TSG-6 knockdown in human ASC. Similarly, for measurement of murine TSG-6, we used commercially available antibodies from R&D systems and validated our ELISA using samples from TSG-6−/− mice-derived murine ASC and comparing our results with those obtained using the ELISA developed by Dr. Mickecz.

ASC have been shown to attenuate inflammation in multiple disease models. Therefore, the next step to study the role of TSG-6 in ASC activities is to assess the performance of ASC in such models after TSG-6 is knocked down. Inflammation arises when large numbers of host cells die in an ischemic attack, such as critical limb ischemia or myocardial infarction. If not properly resolved, it may lead to a vicious cycle of aggravated tissue damage accompanied by escalating excessive inflammation. We have previously established a murine hindlimb ischemia model and witnessed enhanced blood flow recovery and neovascularization as a result of ASC injection, largely due to potent paracrine factors from cells, such as vascular endothelial growth factor (VEGF) and
hepatocyte growth factor (HGF).\textsuperscript{102, 116, 117} In light of the newly identified anti-inflammatory cytokine TSG-6 in the secretome of ASC, we set out to investigate the role of TSG-6 as mediators of the effect of ASC in ameliorating ischemia-induced tissue damage. We also included blood reperfusion as an endpoint to better determine whether TSG-6 may contribute to its improvement either via direct angiogenic effect or via limiting tissue damage at an early stage of injury.

3.2 Materials and Methods

Cell culture

Inguinal adipose tissues of 8-12 weeks old C57BL/6 mice or C.129S6-Tnfaip6\textsuperscript{tm1Cful}/J (TSG-6\textsuperscript{-/-}) mice (gifted by Dr. Katalin Mikecz, Rush University) were isolated and digested at 37°C in PBS containing 2% bovine serum albumin (BSA) and 2 mg/ml collagenase (collagenase A, Roche) for 15-20 min. After filtration through 40 µm nylon filter mesh (BD Falcon) and centrifugation, isolated cells were suspended in medium, counted with a hemocytometer, plated at 5 X 10\textsuperscript{4} cells/ml in 75 cm\textsuperscript{2} tissue culture flasks, and cultured at the presence of EGM2MV medium (Lonza) supplemented with 10% FBS. The cells were daily observed under an inverted phase-contrast microscope and were passaged after 70-80% confluence. The culture media was changed every two days.

Animals

For experiments evaluating limb necrosis as primary endpoints, BALB/c mice (8-10 weeks old, mixed-gender, Jackson Labs) were used because this strain is most susceptible to limb necrosis after femoral artery ligation (FAL).\textsuperscript{118} To test the efficacy of murine ASC and involvement of TSG-6, 24 hrs after FAL, vehicle (PBS) or 2.5 X 10\textsuperscript{5} murine ASC from wild type C57BL/6 or TSG-6\textsuperscript{-/-} mice were administered through bolus intramuscular injections at 2 sites in gastrocnemius muscle of the ischemic leg. To determine the efficacy of human ASC and involvement of TSG-6, 2 hrs after FAL, vehicle or 5 X 10\textsuperscript{5} human ASC not transfected, transfected with TSG-6 siRNA, or with scramble control were administered through intravenous bolus injection. For experiments evaluating
limb distal vascular perfusion as endpoints, NOD.Cg-Prkdcscid IL2rgtmlwlj/SZJ (NSG) mice (8-10 weeks old, female, Indiana University Simon Cancer Center) were used because their rate of blood reperfusion after FAL was less variable. On post-surgical day 1, vehicle or 1 X 10⁶ human ASC not transfected, transfected with TSG-6 siRNA, or with scramble control were administered through intravenous bolus injection. Mice were housed in an environmentally controlled room and were fed chow and water ad libitum. The care of mice complied with the National Research Council’s Guide for the Care and Use of Laboratory Animals. All protocols were approved in accordance with the Committee on Animal Research at Indiana University.

ELISA for Human TSG-6

TSG-6 protein levels in medium from hTNFα-treated hASC were determined by ELISA as previously described with modifications.⁵⁴ A 96-well plate (Maxisorp; Nunc) was coated overnight at 4°C with 50 µl of 10 µg/ml monoclonal antibody specific for TSG-6 (clone A38.1.20; Santa Cruz Biotechnology, Inc.) in 0.2 M sodium bicarbonate buffer (pH 9.2). Plates were washed with PBS after this and all subsequent steps. Nonspecific sites were blocked with 0.25% (w/v) BSA in PBS/0.05% (v/v) Tween (blocking buffer) for 1 hr at room temperature. Samples of 50 µl or standards of recombinant human TSG-6 protein (R&D Systems) in dilution buffer were added and incubated for 2 hrs at room temperature, followed by 50 µl/well of 0.5 µg/ml biotinylated anti-human TSG-6 (R&D Systems) in blocking buffer for 2 hrs at room temperature. Bound antibody was detected by incubation for 20 min with streptavidin-horseradish peroxidase (HRP) (R&D Systems), diluted 1: 200 in PBS and then with substrate solution (R&D Systems) for 20 min. Absorbance at 450 nm and 584 nm was measured by spectrophotometer (SpectraMax M5; Molecular Devices).
ELISA for Murine TSG-6 (ELISA_X)

This ELISA was newly developed using combinations of commercially available antibodies. A 96-well plate (Maxisorp; Nunc) was coated overnight at room temperature with 50 µl of 10 µg/ml monoclonal antibody specific for TSG-6 (clone A38.1.20; Santa Cruz Biotechnology, Inc.) in 0.2 M sodium bicarbonate buffer (pH 9.2). Plates were washed with PBS after this and all subsequent steps. Nonspecific sites were blocked with 0.25% (w/v) BSA in PBS/0.05% (v/v) Tween (blocking buffer) for 2 hrs at room temperature. Samples of 50 µl or standards of recombinant murine TSG-6 protein (R&D Systems) in dilution buffer were added and incubated for 2 hrs at room temperature, followed by 50 µl/well of 0.5 µg/ml biotinylated anti-mouse TSG-6 (R&D Systems) in blocking buffer for 2 hrs at room temperature. Bound antibody was detected by incubation for 20 min with streptavidin-HRP (R&D Systems), diluted 1: 200 in PBS and then with substrate solution (R&D Systems) for 20 min. Absorbance at 450 nm and 584 nm was measured by spectrophotometer (SpectraMax M5; Molecular Devices).

Alternative ELISA for Murine TSG-6 (ELISA_M)

This ELISA was developed by Dr. Katalin Mikecz in Rush University described in more detail in a recent publication. Briefly, a 96-well plate (Maxisorp; Nunc) was coated overnight at room temperature with 50 µl of 5 µg/ml NG3 antibody (gifted by Dr. Mikecz) in 0.2 M sodium bicarbonate buffer (pH 9.2). Plates were washed with PBS after this and all subsequent steps. Nonspecific sites were blocked with 1% (w/v) BSA in PBS (blocking buffer) for 2 hrs at room temperature. Samples of 50 µl or standards of recombinant murine TSG-6 protein (gifted by Dr. Mikecz) in dilution buffer (PBS/1%BSA/0.05%NaN₃) were added and incubated for 2 hrs at room temperature, followed by 50 µl/well of 0.5 µg/ml biotinylated NG8 antibody (gifted by Dr. Mikecz) in blocking buffer for 2 hrs at room temperature. Bound antibody was detected by incubation for 20 min with streptavidin-HRP (R&D Systems), diluted 1: 200 in PBS and then with substrate solution (R&D Systems) for 20 min. Absorbance at 450 nm and 584 nm was measured by spectrophotometer (SpectraMax M5; Molecular Devices).
Transfection of Human ASC with TSG-6 siRNA

A frozen vial of $1.0 \times 10^6$ passage 2 hASC was thawed, and plated at 200 cells/cm$^2$ in 150 mm flasks with 30 ml growth medium (EGM2MV; Lonza) lacking antibiotics. Once 40% confluent, cells were transfected with siRNA for TSG-6 (Santa Cruz Biotechnology) or scramble control (Control siRNA-A; Santa Cruz Biotechnology) with a commercial kit (Lipofectamine RNAiMAX reagent; Invitrogen). For each 150 mm flask, a mixture of 36 µl siRNA (10 µM), 45 µl RNAiMAX, and 3 ml transfection medium (OptiMEM I Medium; Invitrogen) was incubated for 20 min at room temperature and then diluted by 10 fold with OptiMEM I medium before adding to the cells. After incubation for 6 hrs, the medium was replaced with 30 ml growth medium (EGM2MV). To confirm knockdown of TSG-6, mRNA and protein of TSG-6 were assayed by real-time PCR and ELISA respectively.

Mouse hindlimb ischemia model

Left femoral artery excision was performed as described previously.$^{116}$ Briefly, mice were anesthetized using 2% isoflurane inhalation, delivered under a constant oxygen flow rate of 1 L/min. A 0.5-1 cm skin incision was made longitudinally on the anterior thigh of the left hind limb. The femoral artery was exposed under a surgical microscope and dissected from the femoral vein and nerve. The femoral artery and visible branches were ligated proximal to the superficial epigastric artery and above the bifurcation of the saphenous and popliteal arteries with 5.0 silk ligatures. The arterial segment between the ligatures was excised (Figure 12A). Incision was closed with a 5.0 polypropylene suture. Mice received 0.05-0.1 mg/kg subcutaneous Buprenorphine HCL for pain relief. Hind limb blood flow was assessed immediately before and after ligation using Laser Doppler Imaging (Figure 12B and C).
Figure 12. Murine hindlimb ischemia model.

(A) Left femoral artery was ligated and excised between the proximal and distal ligations. Cited from JOV (http://www.biolab.cn/plus/view-428230-1.html). (B) To measure blood perfusion to the limb, mice were anaesthetized with both limbs resting on a warm pad (37°C). Blood perfusion was quantified by triplicate Doppler measurements and normalized to the signal from the contralateral normal limb.
Limb Necrosis Score

An eight-point scoring system modified from previous description was used to characterize severity of limb necrosis after femoral artery ligation (FAL): 0 = normal foot color; 1 = foot discoloration, no necrosis; 2 = necrosis of <3 digit nails; 3 = necrosis of 3-5 digit nails; 4 = necrosis of <3 digits; 5 = necrosis of 3-5 digits, foot looks normal; 6 = <40% foot necrosis; 7 = 41-80% foot necrosis; 8 = complete foot necrosis. Schematic and representative images were shown in Figure 13.
Figure 13. Schematic and representative images of limb necrosis scores.
(A) Schematic showing definition of limb necrosis scores from 0 to 8. 0: normal foot color; 1: foot discoloration, no necrosis; 2: necrosis of <3 digit nails; 3: necrosis of 3-5 digit nails; 4: necrosis of <3 digits; 5: necrosis of 3-5 digits, foot looks normal; 6: <40% foot necrosis; 7: 41-80% foot necrosis; 8: complete foot necrosis. (B) Representative images of different scores from this study.
Laser Doppler perfusion imaging

Mice were anesthetized using 1.5% isoflurane anesthesia by facemask, delivered under a constant oxygen flow rate of 1 L/min. A Laser Doppler perfusion imager (Moor Instruments Ltd) was used to measure dermal blood flow in the dorsal calf and footpad on postoperative days 7, 14, 21, 28, 42, 49. Perfusion ratios of the operated over contralateral non-operated hindlimb (averaged over 3 scans) were compared to minimize differences between ambient temperature and lighting. Mice were maintained between 36.8° and 37.2°C during scanning with a homeothermic warming pad. Image analysis software (Laser Doppler Perfusion Measure, V3.08, Moor Instruments) was used to calculate the limb mean flux units, which represents a quantitative analysis of tissue perfusion on a scale of 0 to 1000. Limb perfusion was expressed as the ratio of the Flux value of the ischemic limb relative to the value of the contralateral limb (Flux Ratio).

Statistical Analyses

Data are shown as mean ± SEM. Comparisons between two groups were made with the use of ANOVA and Newman-Keuls post-hoc analysis. A p-value < 0.05 was considered significant.

3.3 Results

ELISA for human TSG-6

The only hTSG-6 ELISA based on commercially available antibodies is the one published by Lee et al in 2009.54 According to the standard curve in their protocol, the ELISA should be sensitive enough to detect < 0.1 ng/ml rhTSG-6 (Figure 14A). We followed the protocol but found the background noise was so strong at low dose range that the minimum detection dose was as high as 16.2 ng/ml (Figure 14B). Based on the shape of the curve, we chose 62.5-500 ng/ml as the optimal range for the linear standard curve in our later ELISA for human TSG-6. This ELISA was also specific enough to distinguish human and murine TSG-6 even at lowest detectable dose (Figure 14C). One potential pitfall with this
ELISA, however, is that it can be interfered easily by serum proteins through nonspecific competitive binding (Figure 14D). Therefore, for *in vitro* cell culture samples, as long as samples (such as culture medium) were harvested in serum-free conditions, detection of human TSG-6 protein would not be affected.
Figure 14. ELISA for human TSG-6 protein.

(A) Standard curve generated by Prockop lab; (B) Using recombinant hTSG-6 protein from R&D systems, the minimum detection dose (MDD) of hTSG-6 ELISA was 16.2 ng/ml. The optimal detection range was 50-500 ng/ml. (C) The ELISA was highly specific for human TSG-6 protein with minimum reactivity to murine TSG-6. (D) Serum proteins strongly interfered with the detection of human TSG-6.
Comparison of two different ELISAs for murine TSG-6

In order to quantify murine TSG-6 protein, we developed a new ELISA (ELISA_X) using antibodies purchased from R&D systems. Similar to human TSG-6 ELISA, we also observed significant interference from bovine serum proteins even at concentration of 5% (Figure 15A). We also compared this ELISA to an ELISA developed and newly published (in 2012) by Dr. Katalin Mikecz (ELISA_M). Both methods demonstrated satisfactory linear standard curves ($R^2=0.991$ vs. 0.982) at the dose range of 0.3-20 ng/ml (Figure 15B), however, ELISA_M showed a higher sensitivity to increased doses of mTSG-6, implying higher binding capacity of the antibody to mTSG-6 protein. Notably, only 10% dose of mTSG-6 from Mikecz lab was needed to achieve the same intensity of absorbance (OD value) as mTSG-6 from R&D (Figure 15B vs. 14A). Such difference in activities of standard recombinant proteins from different suppliers would result in different quantity readings of the same sample in later experiments.
Figure 15. ELISA for murine TSG-6 protein.

(A) Absence of serum proteins in dilution buffer is advisable since serum proteins interfere with the detection of murine TSG-6 protein. (B) Standard curves of two different ELISAs for murine TSG-6 protein. Antibodies for ELISA_X were purchased from R&D systems; antibodies for ELISA_M were a kind gift of Dr. Mikcez.
Knocking down TSG-6 in human ASC by siRNA

We have shown in Chapter 2 that inflammatory cytokine TNFα highly upregulates secretion of TSG-6 from human ASC. To understand the importance of this response mechanism to activities of ASC, it is necessary to mute the activation of TSG-6 transcription. For this purpose, human ASC were transfected with TSG-6 siRNA. In parallel plates, cells were incubated with Alexa Fluor 555-labeled dsRNA to verify the efficiency of transfection. After transfection, red fluorescence was found in ~90% of cell nuclei. Correspondingly, expression of TSG-6 mRNA and secretion of TSG-6 protein in the presence of TNFα stimulation both decreased by more than 90% (Figure 16B and C). Although scramble control RNA slightly decreased TSG-6 expression, at protein level, it is not different from un-transfected control (Figure 16B and C). By washing the cells with PBS and incubating for additional 24 hrs with fresh basal medium (EBM2, Lonza), we also demonstrated that the memory of stimulation by TNFα and interference by TSG-6 siRNA remained in hASC for at least 24 hrs after removal of the original treatment (Figure 16D). This allowed us to generate conditioned media from regular and inactivated (TSG-6 siRNA transfected) hASC, and compare their efficacy without being confounded by TNFα and siRNA.
A

ASC

Red: AF555-dsRNA

B

C

control  TSG6 siRNA  scr siRNA

baseline  TNFαX1d

control  TSG6 siRNA  scr siRNA

baseline  TNFαX1d

D

control  TSG6  scr

siRNA

TSG6 (ng/ml)

control  TSG6  scr

siRNA
Figure 16. Knockdown of TSG-6 production from human ASC by siRNA. (A) Human ASC maintained their normal morphology while more than 90% of nuclei had been infiltrated by fluorescently labeled (red) dsRNA. (B & C) Real-time PCR and ELISA confirmed effective knockdown of TSG-6 at both mRNA and protein level. (D) To generate conditioned medium enriched for TSG-6, transfected ASC were stimulated with 20 ng/ml human TNFα for 24 hrs, washed with PBS twice (to remove TNFα), and incubated in fresh EBM2 medium for another 24 hrs before supernatants were harvested. Levels of TSG-6 were confirmed by ELISA. N=3/group. **p<0.01.
Verification of TSG-6 knockout from murine ASC

Deficiency of TSG-6 protein causes infertility in female mice and higher susceptibility to tissue damage from inflammatory diseases such as arthritis. How absence of TSG-6 gene affects fat tissue and cells derived from it remains unknown. To obtain murine ASC with defect in TSG-6 secretion, we harvested peri-renal and subinguinal fat tissue from wild type C57BL/6 mice and TSG-6−/− mice. The yield and morphology of ASC were similar between two groups of mice (Figure 17A and B). To verify functional knockout of TSG-6 gene, murine ASC were stimulated with 20 ng/ml murine TNFα for 24 hrs. Murine TSG-6 in the supernatant was quantified by two different ELISA methods. Both assays showed lower levels of TSG-6 secretion from mASCko compared to mASCwt. Larger difference was seen in ELISA_M (Figure 17 C and D). Interestingly, despite a similarly strong induction of TSG-6 expression at mRNA level (Figure 17E), the productivity of TSG-6 protein was much lower in murine ASC than in human ASC (pg vs. ng).
Figure 17. Murine ASC from wild type (mASC\textsuperscript{wt}) and TSG-6\textsuperscript{-/-} mice (mASC\textsuperscript{ko}). No morphological difference was observed between adipose stem cells (ASC) isolated from wild type (A) and TSG-6\textsuperscript{-/-} mice (B). Based on both ELISA_M (C) and ELISA_X (D), secretion of mTSG-6 from mASC\textsuperscript{ko} was lower than from mASC\textsuperscript{wt} at baseline and upon murine TNF\(\alpha\) (20 ng/ml) stimulation. (E) Transcription of TSG-6 in wild type murine ASC was highly upregulated after 24 hrs of TNF\(\alpha\) (20 ng/ml) treatment.
Effects of murine ASC on limb necrosis and muscle atrophy

Ischemic limb necrosis is a typical *in vivo* inflammation model to assess the anti-inflammatory activities of ASC because the tissue damage largely results from the release of proteases subsequent to excessive inflammation. Symptoms of limb necrosis in BALB/c mice progressed acutely after femoral artery ligation and mostly stopped within the first week. We injected murine ASC intramuscularly 24 hrs after surgery, because a similar population of mesenchymal stem cells, murine marrow stromal cells (BMSC) administered in the same time frame through the same route resulted in significant improvement of limb necrosis in the same strain of mice.\textsuperscript{120} Unexpectedly, murine ASC from wild type mice failed to improve limb necrosis, although fewer cells were injected compared to the previous study (2.5 vs. 10 X 10\textsuperscript{5}) due to limited supply. Interestingly, ASC from TSG-6\textsuperscript{-/-} mice seemed to further aggravate the symptoms (Figure 18A). We also observed large variations in the severity of necrosis (scores from 1 to 6) even within the same treatment group, as reflected by the large standard deviations (Figure 18B). Overall, no statistical difference was observed among all treatment groups. In accordance with the result of necrosis scores, calf muscle atrophy also varies among individuals in each group (Figure 19B). Neither was any significant difference observed.
Figure 18. Effects of murine ASC on limb necrosis.

(A) Progression of limb necrosis after surgery. One day after surgery, BALB/c mice were injected locally with $2.5 \times 10^5$ mASC<sup>wt</sup> or mASC<sup>ko</sup>. Severity of necrosis was rated from 0 to 8 (lowest to highest), as described in Figure 13. (B) Individual variability was observed in all treatment groups. No statistically significant difference was observed among groups. PBS = phosphate buffered saline; mASC<sup>wt</sup> / mASC<sup>ko</sup> = murine ASC from wild type or TSG-6<sup>-/-</sup> mice. Mean ± SEM, n=7/group. NS=non significant.
Figure 19. Effects of murine ASC on calf muscle atrophy.

(A) Calf muscle (gastrocnemius and soleus) from both ischemic and contralateral normal limbs were excised on post-surgical day 8 and weighed. (B) Severity of muscle atrophy was evaluated by calculating the ratio of ischemic vs. normal muscle weight. PBS = phosphate buffered saline; mASC<sup>wt</sup> / mASC<sup>ko</sup> = murine ASC from wild type or TSG-6<sup>−/−</sup> mice. Mean ± SEM, n=7/group. NS=non significant.
Effects of human ASC on limb necrosis

We also attempted using human ASC at higher dose (1 X 10^6/mouse) to ameliorate limb necrosis since mesenchymal stem cells from other sources such as bone marrow and placenta have demonstrated robust cross-species benefits to improve ischemia.\textsuperscript{54, 121} Besides, according to our above data, human ASC secrete more TSG-6 than murine ASC. We also changed the delivery route to intravenous injection instead of intramuscular and the time for injection to 2 hrs instead of 24 hrs after surgery, because human MSC have been shown to secrete TSG-6 \textit{in vivo} after intravenous administration and the efficacy of TSG-6 declined with waiting time after injury occurred.\textsuperscript{54, 55}. However, despite these efforts in improving the study design, we still weren’t able to find any improvement in limb necrosis subsequent to hASC treatment (Figure 20A), most likely due to the large variability within each group as we have seen before (Figure 20B).
Figure 20. Effects of human ASC on limb necrosis.

(A) Progression of limb necrosis after surgery. Two hours after surgery, BALB/c mice were injected i.v. with $1 \times 10^6$ human ASC not transfected (ASC\textsuperscript{NT}), transfected with TSG-6 siRNA (ASC\textsuperscript{TS}), or with control scramble siRNA (ASC\textsuperscript{SC}).

(B) Large individual variability was observed in all treatment groups. No statistically significant difference was observed among groups. Mean ± SEM, n=7/group.
Involvement of TSG-6 in ASC-enhanced blood flow recovery

Compared to necrosis score, laser Doppler imaging of blood flow is a more objective and commonly used method to evaluate the recovery from femoral artery obstruction. We have previously shown that human ASC i.v. 24 hrs after femoral artery ligation improved blood flow recovery in NSG mice. In this study, we followed our routine injection protocol but knocked down TSG-6 in human ASC by siRNA before injection. As expected, regular ASC enhanced blood flow recovery compared to vehicle control and the difference reached statistical significance 2 weeks after surgery (Figure 21A). This therapeutic effect of ASC was lost when cells were transfected with siRNA, which seemed to have a nonspecific effect since TSG-6 siRNA and scramble RNA both weakened the potency of human ASC (Figure 21B).
Figure 21. Effects of human ASC on blood reperfusion of ischemic limb.
(A) One day after surgery, NSG mice were injected i.v. with $1 \times 10^6$ human ASC not transfected (ASC$^{NT}$), transfected with TSG-6 siRNA (ASC$^{TS}$), or with control scramble siRNA (ASC$^{SC}$). (B) Compared to the PBS control, the therapeutic effect of ASC became significant 2 weeks after surgery. No significant improvement was observed in ASC$^{TS}$ or ASC$^{SC}$ group. Mean ± SEM, n=6-7. *$p<0.05$. 
3.4 Conclusion

We successfully optimized human TSG-6 ELISA and established murine TSG-6 ELISA to quantify TSG-6 protein. Afterwards, we verified the effectiveness of knockdown of hTSG-6 in human ASC and reduced secretion of mTSG-6 from murine ASC\textsuperscript{ko}. Although femoral artery ligation did result in limb necrosis and gastrocnemius muscle atrophy in BALB/c mouse strain, the individual variability was too high to detect any difference between regular ASC and ASC with TSG-6 knocked down. TSG-6 siRNA had no specific effect on blood flow recovery after femoral artery occlusion.

3.5 Discussion

The advantage of using murine ASC is that TSG-6 could be permanently knocked out (or down depending on ELISA protocols). The disadvantage, however, is that cell supplies may be limited because murine ASC are much more difficult to grow and senesce at early passages.

Using same murine TSG-6 antibodies but different sources of standard recombinant murine TSG-6 protein, we saw a 10-fold difference in the dose of TSG-6 protein required to produce the same OD values (absorbance). This underlines the importance to factor in the source of standard proteins when comparing TSG-6 quantities among studies. From this perspective, an ELISA based on commercially available reagents enables easier sharing and comparisons of results from different studies. Similarly, the discrepancy between our hTSG-6 standard curve and the one from Prockop lab using the same protocol may result from different batches of standard TSG-6 proteins.

Intriguingly, the response of limb muscles to femoral artery ligation varies dramatically among different mouse strains. Based on literature and our own experience, skin discoloration or limb necrosis seldom manifested in C57BL/6 and NSG strain, whereas the severity of these symptoms varies among individuals in A/J, CSS, and nude mice.\textsuperscript{118} We chose BALB/c mice because they not only develop severe limb necrosis but also respond to local injections of BMSC.\textsuperscript{120} That said, their symptoms of limb necrosis also varied between grade
1 and 3 (on a scale of 0-4). To further reduce potential variability, we graded the severity of necrosis from 0 to 8 instead of commonly used grading of 0 to 4. Nevertheless, large variability of necrosis score persisted and no benefit from local injections of murine ASC or systemic injections of human ASC were recorded.

The large individual variability of limb necrosis in BALB/c mice renders limb necrosis score a less sensitive endpoint to assess the efficacy of treatment. However, one might still be able to detect a potential therapeutic effect if optimizing conditions that would enhance the magnitude of the effect. Notably, in the previous BMSC study, the window of cell delivery was changed from immediately after femoral ligation to 24 hrs post-ligation because the former failed to improve flow recovery. This example underlined the importance of pharmacodynamic factors to the overall effect of injected cells. Therefore, to maximize any potential anti-inflammatory effect ASC may have, it is essential to optimize injection parameters such as cell passage, dosage, injection site, etc.

In addition, another study pointed out that the stability of limb necrosis also depends on the anatomic location of ligations. Specifically, more variations were observed when the femoral artery was ligated proximal to the branch of popliteal artery (as was done in this study), possibly due to the variability in compensations from collateral circulation. The stability of limb necrosis can be largely improved by extending the resected segment to include popliteal artery branch, therefore blocking collateral blood flow compensations.

Alternatively, other more sensitive inflammatory disease models may be employed to investigate the involvement of TSG-6 in the therapeutic effect of ASC. In the next chapter we used a murine model of cigarette smoking, which has been known to cause systemic inflammation.
Chapter 4. Rejuvenation of Smoking-induced Bone Marrow Progenitor Exhaustion: Moving from Adipose-Derived Stem Cells to Their Secretome

4.1 Introduction

Proinflammatory cytokines such as TNFα and IFNγ released in inflammation have been shown to potently suppress hematopoiesis. The bone marrow (BM) has traditionally been viewed as a hematopoiesis organ to produce blood cells for daily physiological needs. In recent years, it has been increasingly recognized as a residence niche for stem and progenitor cells that can be mobilized to contribute to homeostasis and tissue repair of extra-medullary organs. Depletion or even functional exhaustion of bone marrow reservoir of stem / progenitor cell has been correlated with worse prognosis and higher morbidity of numerous diseases including coronary heart disease, chronic obstructive pulmonary disease (COPD), diabetes, etc.

Cigarette smoking (CS) is a major risk factor for atherosclerotic vascular disease, COPD, and cancer. In addition, it also has well-known adverse effects on other organ systems including kidney, liver, reproduction, and innate immunity. In contrast, the effect of CS on bone marrow stem/progenitor cells has been much less explored. Previous studies revealed a reduction of hematopoietic progenitors after mice were exposed to CS for 3 weeks. This echoes with the clinical observations that exposure to CS or its active ingredient nicotine correlates with worse outcomes of patients receiving HSC transplantation and inferior colonization of fetal bone marrow by HSC. The progression of and countermeasure for this CS-induced bone marrow dysfunction remain unknown.

Adipose stem or stromal cells (ASC), which reside in the “non-adipocyte” fraction of the adipose tissue, represent a particularly feasible form of cell therapy for tissue repair and rescue from injury due to the easy access through liposuction. Several reports have described their capability to facilitate normal hematopoiesis, much resembling their counterpart marrow stromal cells. They have not been tested, however, as a countermeasure to correct bone marrow progenitor dysfunctions, including those caused by cigarette smoking.
Moreover, after intravenous administration, these cells usually home to the lung and release various paracrine factors contributing to the protection and recovery of injuries in distant organ systems.\textsuperscript{117, 143, 144}

In a previous chronic CS-induced murine emphysema model, we serendipitously observed a marked reduction of BM progenitors in addition to typical emphysematous abnormalities in the lung, both of which can be corrected by periodic injection (i.v.) of murine ASC.\textsuperscript{145} In this study, we shortened the exposure time to trace the beginning of CS-induced BM dysfunction. We used xenograft human ASC (hASC), which translate more easily into future clinical therapy, to counteract the effect of CS. We also, successfully identified specific cytokine TSG-6, previously not been recognized in the ASC secretome, to be the key factor responsible for the potent activity of hASC in this model.

### 4.2 Materials and Methods

#### Cell culture

Human ASC were isolated and cultured as described in Chapter 2.

#### Animals

Animal studies were approved by the Animal Care and Use Committee of Indiana University. \textit{In vivo} CS exposure was performed as previously described.\textsuperscript{146} Briefly, mice were exposed to 11\% mainstream and 89\% side-stream smoke from reference cigarettes (3R4F; Tobacco Research Institute) using a Teague 10E whole body exposure apparatus (Teague Enterprise). The exposure chamber atmosphere was monitored for total suspended particulates (average, 90 mg/m\textsuperscript{3}) and carbon monoxide (average, 350 ppm). For short-term exposure, C57BL/6 (female, age 6-8 wks, Jackson Laboratories) mice were exposed to CS for 1 day or 3 days (5 hrs/day). A bolus dose of 3 $\times$ 10\textsuperscript{5} hASC was injected (tail vein, i.v.) before 1-day CS or after day 2 of 3-day CS. PBS was used as a control vehicle. For intermediate-term exposure, C57BL/6 were exposed to CS for 7 weeks (5 hrs/day, 5 days/week) and received weekly injection (i.v.) of 3 $\times$ 10\textsuperscript{5} hASC. For long-term exposure, NSG mice were exposed
to CS for 6 months (5 hrs/day, 5 days/week) and received weekly injection (i.v.) of $2.5 \times 10^5$ hASC starting from 3rd month of CS (Figure 22). In all CS experiments, mice were euthanized and lungs were processed as previously described the day after the last day of CS exposure.$^{147}$
Figure 22. Schedule of *in vivo* cigarette smoking (CS) exposure and hASC administrations.

C57BL/6 mice were exposed to short or intermediate-term CS and received either bolus or weekly injections of $3 \times 10^5$ human ASC (hASC) or vehicle control. NSG mice were exposed to long-term CS and received weekly hASC ($2.5 \times 10^5$ / mouse) after smoking for 2 months.
CS Extract Preparation

An aqueous CS extract was prepared from filtered research-grade cigarettes (1R3F) from the Kentucky Tobacco Research and Development Center at the University of Kentucky. A stock (100%) CS extract was prepared by bubbling smoke from two cigarettes into 20 ml of basal culture medium (EBM2, Lonza) at a rate of one cigarette per minute to 0.5 cm above the filter, as previously described.\textsuperscript{146} The extract’s pH was adjusted to 7.4, followed by filtration (0.2 mm, 25 mm, Acrodisc) and used in cell culture experiments within 20 min. A similar procedure was used to prepare the control extract, replacing the CS with ambient air.

Clonogenic Progenitor Cell Assay

Bone marrow from mice were assessed for granulocyte-macrophage colony-forming unit (CFU-GM), erythroid burst-forming unit (BFU-E), and multipotential granulocyte, erythroid, monocyte, megakaryocyte colony-forming units (CFU-GEMM) progenitor cells, as previously described.\textsuperscript{148,149} In short, BM cells were flushed from both femurs, and plated at 5 X 10⁴ cells/ml in 0.9% methylcellulose culture medium with 30% FBS (Hyclone), and 1 U/ml recombinant human erythropoietin (Epo, Amgen Corp), 50 ng/ml recombinant murine stem cell factor (rmuSCF, R&D Systems), 2 mM glutamine (Cambrex Bio Science), 10⁻⁴ M 2-mercaptopoethanol (Fisher Scientific), 0.1 mM hemin (Sigma-Aldrich) and 5% vol./vol. pokeweed mitogen mouse spleen cell conditional medium. For \textit{in vitro} CSE toxicity assay assessing CFU-GM only, 5 X 10⁴ cells were plated and cultured in 0.3% methylcellulose culture medium in the presence of 50 ng/mL rmuSCF and 10 ng/mL recombinant murine GM-CSF (rmuGM-CSF, R&D Systems). Absolute numbers of progenitors were calculated from the nucleated cellularity per femur and the number of colonies formed per number of cells plated. The percentage of progenitors in the S phase of the cell cycle was estimated by use of the tritiated thymidine kill technique, as described previously.\textsuperscript{149} Colonies were scored by two blinded readers (Figure 23) after 7 days of incubation at 37°C, in lowered (5%) O₂ and 5% CO₂. The high specific
activity tritiated thymidine kill assay allows an estimate of the cell cycling status of progenitors by analysis of the percent progenitors in S-phase at time cells were removed from mice and plated.
Figure 23. Correlation between 2 blinded readers for the CFU assays.
CFU-GM colonies in plates from different experiments were scored by two blinded readers for colony numbers.
Phenotypic Analysis of Bone Marrow Stem and Progenitor Cells

The following phenotypically identified stem and progenitor cell populations were assessed in the bone marrow (BM): lineage$^{-}$Sca$^+$c-Kit$^+$ cells (LSK, enriched for hematopoietic stem cells), multi-potent progenitors (CD34$^+$CD135$^+$ LSK), common lymphoid progenitor (CLP; Lin$^{-}$Sca$^+$c-Kit$^+$IL$^-7R\alpha^+$), common myeloid progenitor (CMP; Lin$^{-}$Sca$^-$c-Kit$^+$CD34$^+$FcγR$^{-}$/low), and granulocyte/macrophage progenitor (GMP; Lin$^{-}$Sca$^{-}$c-Kit$^+$CD34$^+$FcγR$^{+/high}$). BM was collected and stained with antibodies to surface markers previously described. Anti-mouse antibodies c-Kit, Sca1, FcγR, IL7Rα, Lineage Cocktail, and isotype controls were purchased from BD Biosciences. Anti-mouse CD34 antibody was purchased from eBioscience and Sca1 was also purchased from BioLegend. Data was collected from the samples using a LSR II (BD Biosciences) instrument and BD FACSDiva software (BD Biosciences), and was analyzed using Flowjo software (Tree Star).

Cell Cycle Analysis of Bone Marrow Sca1+ Progenitor Cells

Total mouse bone marrow cells were stained with FITC-Sca1 antibody (BD Biosciences) and sorted for Sca1$^+$ cells on Reflection II (BD Biosciences). Cell cycling staining was performed as previously described. Briefly, cells (5 X 10$^3$) were washed with PBS containing 0.1% BSA and incubated with 5 mg/ml of RNase A (DNase free) and 50 mg/ml propidium iodide (PI) for 15 min at room temperature. The percentages of cells in various phases of the cell cycle were measured with a FACSCalibur flow cytometer (BD Biosciences).

Histology of Bone Marrow Cellularity in Femur

The right femur of each mouse was harvested and fixed in 4% phosphate buffered formalin (PBF) for 48 hrs, after which it was demineralized in a solution of 10% EDTA and 4% PBF (7:3 ratio) at 4°C with agitation for a week. Femurs were then embedded in paraffin and longitudinally sectioned at 4 µm. Two slides from each femur were stained with hematoxylin and eosin (H&E) for microscopic
examination. To stain proliferative cells, one slide from each femur was stained with rabbit anti-mouse Ki-67 antibody (Abcam).

RNA Extraction and Quantitative Real-Time PCR

Whole lungs were perfused free of blood via right ventricular perfusion with 10 ml warmed saline, rapidly excised en bloc, blotted, and snap-frozen in liquid nitrogen. Total RNA was extracted from lung homogenates using the RNeasy mini kit (Qiagen) following manufacturer’s instructions. Reverse transcription with High-Capacity cDNA Archive Kit (Applied Biosystems) and triplicate real-time PCR with SYBR Green Master Mix (Applied Biosystems) and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) internal control were carried out as previously described.152
**Table 1. The sequences of the polymerase chain reaction (PCR) amplification primers**

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Transfection of hASC with TSG-6 siRNA

Knockdown of TSG-6 by siRNA and verification of TSG-6 levels by ELISA were described in Chapter 2.

Statistical Analyses

Data are shown as mean ± SEM. Comparisons between two groups were made with the use of ANOVA and Newman-Keuls post-hoc analysis. A p value of < 0.05 was considered significant.

4.3 Results

Acute reduction and slow recovery of clonogenic BM progenitor cells after CS and effect of hASC

Khaldoyanidi first noticed the decline of bone marrow myeloid progenitors when they exposed BALB/c mice to CS for 3 weeks. In our study using C57BL/6 mice, we surprisingly found this adverse effect of CS occurred much more acutely – CFU-GM started declining up to 35% after only 1 day of CS exposure, which can be prevented by a bolus injection (i.v.) of $3 \times 10^5$ human ASC before smoking (Figure 24A). This acute effect was less prominent on BFU-E and CFU-GEMM (Figure 24B and C).
Figure 24. ASC prevent CFU-GM from acute suppression caused by one day of cigarette smoke exposure.

C57BL/6 mice were injected with $3 \times 10^5$ human ASC i.v. before exposure to air or smoke for 1 day. CFU-GM: colony forming unit–granulocyte, monocyte; BFU-E: burst forming unit–erythroid; CFU-GEMM: colony forming unit–granulocyte, erythrocyte, monocyte, and megakaryocyte. Mean ± SEM, n=3 mice/group. *p<0.05, **p<0.01.
After extended exposure (3 days), CFU-GM counts continued to decline – by approximately 40%; and the toxicity of CS extended to other CFUs such as BFU-E and CFU-GEMM, which decreased by approximately 55% and 50%, respectively (Figure 25). These different types of progenitors not only differ in their sensitivity to CS but also in their rate of post-CS recovery. For instance, CFU-GM and CFU-GEMM were not fully recovered after cessation of CS for twice as long as exposure time. Given the hematopoiesis-supporting nature of adipose stem cells and our previous success using them to reverse systemic CS toxicity in chronic smoking mice, we tested bolus injection (i.v.) of xenograft human ASC as a countermeasure in this acute smoking model. To mimic treatment window for patients with smoking history, cells were administered in the middle of the entire CS period (after day 2 of 3-day CS exposure). Remarkably, ASC restored all 3 lineages of progenitors to levels even higher than those after 7 days of recovery, much close to the nonsmoker level (Figure 25).
Figure 25. Self-recovery of mouse bone marrow hematopoietic progenitor cells after 3-day CS and effect of ASC.

C57BL/6 mice were exposed to air or cigarette smoke (CS) for 3 days then rested for 1-7 days (D1-D7) for recovery. One additional group received a bolus 3 X 10^5 human ASC i.v. after day 2 and bone marrow were harvested one day after last CS exposure. CFU-GM: colony forming unit– granulocyte, monocyte; BFU-E: burst forming unit–erythroid; CFU-GEMM: colony forming unit–granulocyte, erythrocyte, monocyte, and megakaryocyte. Mean ± SEM, n=3-6 mice/group. 

a_p<0.05 (vs. air), b_p<0.05 (vs. day 1).
Exhaustion of phenotypic stem and progenitor cells with prolonged exposure and effect of hASC

Notably, the rapid loss of functionally defined (based on clonogenic capacity) progenitors occurred latently without changes of total cellularity in the bone marrow cavity (Figure 26 and 27A) and couldn't be detected in early phase (3 days) by flow cytometry based on commonly used phenotype markers (Figure 27). The distinct contrast between dramatic change in clonogenic capacity and lack of change in phenotype identity indicate that while phenotypic markers may help distinguish progenitor cells from mature cells, they offer no information on the functionality and physiological status of BM stem and progenitor cells. This speculation directed our later efforts to studying functionality of progenitor cells using colony-forming-unit assays. In addition, it also supports the notion that changes in functionality of BM progenitors precede changes in their phenotype. To prove this hypothesis, we extended the exposure time to 7 weeks. As expected, we observed a significant reduction (by early 60%) of Lineage⁻ Sca1⁺ c-Kit⁺ bone marrow cells, which are enriched with stem and progenitor cells. Consistent with findings in the acute model, weekly injection of human ASC was able to fully reverse the damage to the level of nonsmoking mice (Figure 28B). These data demonstrate that ASC were able to protect BM stem and progenitors from CS-induced depletion, possibly by reversing the exhausted clonogenic capacity caused by CS at early stage.
Figure 26. H&E staining of distal femur showing lack of change in bone marrow cavity structure and cell distribution after treatment.

Right femurs were harvested from C57BL/6 mice exposed to 3-day smoking, demineralized, paraffin-embedded, longitudinally sectioned, and stained with hematoxylin and eosin (H&E). Images showed the distal end of each femur.
Figure 27. Phenotypic progenitors remain unchanged after 3-day CS.

(A) Settings for multi-color flow analysis of phenotypic hematopoietic stem and progenitor cells. MPP: Multi-Potent Progenitors; GMP: Granulocyte-Macrophage Progenitors; CMP: Common Myeloid Progenitors; CLP: Common Lymphoid Progenitors. (B) C57BL/6 mice were exposed to 3-day ambient air (AC) or cigarette smoke (CS). No difference was observed for various phenotypic progenitors (n=6/group).
Figure 28. Prolonged CS-induced depletion of phenotypic hematopoietic stem / progenitor cells and effects of human ASC.

After extended exposure to CS (from 3 days to 7 weeks, 5 hrs/day), although total bone marrow cellularity remains unchanged (A), Lin-Sca1+c-Kit+ cells (enriched for hematopoietic stem and progenitor cells) significantly reduced (B). For 3d-smoking mice, 3 X 10^5 hASC were injected i.v. after day 2. For 7wk-smoking mice, 3 X 10^5 hASC was injected i.v. weekly. Total bone marrow cells were flushed from both femurs and counted using a Hemavet (Drew Scientific) instrument (n=11/group for 3d-smoking, 3/group for 7wk-smoking). LSK were quantified by flow cytometry (n=3/group). Mean ± SEM. *p<0.05.
Absence of Injected hASC in Mouse Bone Marrow

In search for mechanisms underlying therapeutic effects of ASC observed above, our first theory was that the effect came from locally engrafted ASC. After all, in certain disease models, systemically delivered hASC were able to home to the site of injury and contribute to tissue regeneration. If this indeed occurred also in smoking mice, the best chance to capture engrafted hASC in mouse bone marrow is to collect total mouse bone marrow and stain for human-specific antigens. Therefore, we isolated total bone marrow cells from immunotolerant NSG mice (to exclude the possibility of immune rejection to xeno-transplanted cells) receiving weekly hASC for 4 months (to accumulate more engrafted hASC) and plated them on plastic surface. After 7 days, medium was replaced to remove unattached hematopoietic cells and the attached cells (hASC adhere to plastic surface) were stained with anti-human nuclei Ab. Surprisingly, we were not able to find any human cells (Figure 29), which suggests hASC were more likely to function remotely through paracrine factors.
Figure 29. Absence of human cell engraftment in NSG mice receiving intravenous hASC.

NSG mice were exposed to 6 months of cigarette smoking and administered i.v. with human ASC weekly since the 3rd month. Total bone marrow cells were harvested from 2 femurs and plated on plastic in DMEM/10%FBS. Cells were stained with mouse anti-human nuclei Ab. Human ASC were used as positive control. Murine endothelial cells were used as negative control.
ASC respond to CS-induced host inflammatory cytokines by secretion of TSG-6.

We have previously shown that intravenously injected ASC were transiently trapped in the lung,\textsuperscript{146} where they may be exposed to inflammatory mediators produced in lungs exposed to CS\textsuperscript{153}. Given that our data described in chapter 2 showed secretion of TSG-6 from ASC was triggered by environmental inflammatory signals, we measured the inflammatory cytokine secretion in the lung and the TSG-6 expression in the lungs exposed to CS at a time when ASC were found in the lung. Increased release of proinflammatory cytokines is a hallmark of CS-related pathophysiology.\textsuperscript{153} As expected, we observed a marked increase of TNF\(\alpha\) (by 4.1 fold) and IL1\(\beta\) (by 3.9 fold) transcripts in the lungs exposed to CS (Figure 30A). In this context, a recent study also demonstrated that stromal cells from bone marrow trapped in the lung after i.v. delivery were activated by TNF\(\alpha\) to secrete anti-inflammatory cytokine TSG-6.\textsuperscript{54} Indeed, using human-specific primers for TSG-6 gene, we detected high levels of human TSG-6 transcripts in lung from mice receiving hASC (Figure 30B). To further elucidate this feedback mechanism of hASC responding to host pro-inflammatory signals, we measured secretion of TSG-6 from ASC \textit{in vitro} cells were treated with TNF\(\alpha\) or IL1\(\beta\). After incubation with 20 ng/ml human TNF\(\alpha\) or IL1\(\beta\) for 24 hours, TSG-6 secretion increased from undetectable levels to over 5.0-8.7 ng/10\(^3\) cells in 24 hrs (Figure 31A). Moreover, in parallel experiments, mouse TNF\(\alpha\) or IL1\(\beta\) demonstrated equivalent stimulatory effects on human ASC (Figure 31B), suggesting a dynamic cross-species interaction between the host environment and injected xenograft human cells. This activated TSG-6 secretion from ASC in the context of smoking-induced lung inflammation highly warrants further investigation into potential role of this protein in mitigating CS toxicity.
Figure 30. Increased murine inflammatory cytokines and human TSG-6 transcripts in lungs of smoking mice.

(A) Expression of both murine TNFα and IL1β were up-regulated in the lung after mice were exposed to 3-day cigarette smoking (CS) compared to air control (AC); (B) minimum human-specific TSG-6 mRNAs were detected in untreated mouse lungs, which markedly increased 40 hrs after intravenous injection of human ASC. Mean ± SEM, n=3. *p<0.05; **p<0.01.
Figure 31. Both human and murine TNFα and IL1β activate human ASC to secrete TSG-6.

(A) In vitro, secretion of TSG-6 protein from human ASC is low at baseline but strongly increased after stimulation of human pro-inflammatory cytokines (TNFα and IL1β, but not IFNγ); (B) secretion of TSG-6 from hASC can be activated similarly with cross-species mouse pro-inflammatory cytokines. Mean ± SEM, n=3. **p<0.01.
TSG-6 directly antagonizes myelotoxicity of smoking

TSG-6 may attenuate the myelotoxicity of smoking through two mechanisms: one is to directly block effects of toxins (including pro-inflammatory cytokines such as TNFα) on bone marrow progenitors, the other is to reduce the amount of toxins resulted from smoking. To test the first possibility, we established an *in vitro* model using cigarette smoke extract as a substitute for the mixture of dissolved circulatory compounds derived from smoke. We only looked at CFU-GM colonies because they consist of 90% of all colonies and is a more accurate indicator. In accordance with *in vivo* observations, CFU-GM decreased by 75.1% after treatment of 1.5% CSE for 7 days. This direct toxicity of smoke-derived compounds can be fully antagonized by co-cultured hASC or supplemented ASC-conditioned media, and partially attenuated by recombinant TSG-6 protein (0.5 µg/ml), but not by conditioned media from ASC in which TSG-6 secretion was silenced (Figure 32). These data support the novel application of TSG-6, in addition to its anti-inflammatory functions, as an antagonist against myelotoxins such as those derived from smoke. They also highlighted the critical role TSG-6 played in mediating antagonistic effects of ASC secretome against toxicity of CSE *in vitro*.
Figure 32. Protection of GM-CFU by ASC, ASC conditioned media, and TSG-6 from toxicity of cigarette smoke extract (CSE) *in vitro*.

Mouse bone marrow cells were treated *in vitro* with 1.5% CSE with or without ASC / ASC-CM / TSG-6 in methylcellulose medium supplemented with FBS, mGM-CSF (10 ng/ml) & mSCF (50 ng/ml) for 7 days. Mean ± SEM, n=3/group. \(^a\)p<0.05, (vs. non-treated control); \(^b\)p<0.05, vs. (CSE + vehicle). ASC\(^{NT}\)=Non-transfected ASC; ASC\(^{TS}\)=TSG-6 siRNA-transfected; ASC\(^{SC}\)=scramble RNA-transfected.
TSG-6 is a key mediator for myeloprotective effects of hASC

To determine the role of TSG-6 in the myeloprotective effect of hASC in vivo, equal amounts (3 \times 10^5) of hASC either not transfected, or transfected with TSG-6 siRNA or scramble siRNA were administered after day 2 of 3-day CS exposure. Analysis of bone marrow revealed that non-transfected or scramble siRNA-transfected hASC were still capable of reversing the significant decrease of CFU-GM and BFU-E subsequent to smoking, while hASC transfected with TSG-6 siRNA almost completely lost their therapeutic effect (Figure 33A and B). Changes of CFU-GEMM were not statistically significant due to high variability but had the same trend as CFU-GM and BFU-E (Figure 33C). These results suggest that TSG-6 is a key mediator of myeloprotective functions of hASC in vivo.
Figure 33. Myeloprotective effects of hASC were lost after TSG-6 knockdown.

C57BL/6 mice were exposed to 3-day CS and injected i.v. with $3 \times 10^5$ hASC either not transfected (ASC<sup>NT</sup>), or transfected with TSG-6 siRNA (ASC<sup>TS</sup>) or with control scramble siRNA (ASC<sup>SC</sup>) after day 2. Mean ± SEM, n=6/group.  \textsuperscript{a}p<0.05 (vs. AC); \textsuperscript{b}p<0.05, (vs. vehicle).
Effects of hASC can be reproduced by hASC-conditioned medium and TSG-6

Conditioned media with active ingredients have numerous advantages over producer cells per se from application perspectives. Cell-free conditioned media also help avoid potential side effects from rejections to allogeneic or xenograft cell transplantation by the host environment. Given the importance of trophic factors, including TSG-6 to the effect of hASC, we next attempted to replace cells with their conditioned media. As expected, conditioned media from TNFα-activated regular hASC fully recapitulated the therapeutic effect of producer cells. In contrast when TSG-6 was knocked down in hASC, the efficacy of the derived conditioned media was also reduced, which could be compensated by supplementing additional exogenous recombinant TSG-6 protein. These data highlighted TSG-6 as a critical ingredient in ASC-conditioned media and therefore its potential usefulness as an indicator for the quality control of ASC conditioned media. Notably, the concentration of recombinant TSG-6 protein alone necessary to achieve the same effect as conditioned media was at least 10 fold higher than the concentration of endogenous TSG-6 present in conditioned media (as measured by ELISA) (Figure 34A). This indicated that either the recombinant TSG-6 protein, produced in a mouse myeloma cell line, was of weaker potency than that naturally secreted by human ASC due to different folding, for example, or that there are other yet unknown in the conditioned media that synergize with TSG-6 to enhance its efficacy (Figure 34A).
### CFU-GM

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Figure 34. Effects of ASC against myelotoxicity of cigarette smoke can be reproduced by ASC conditioned medium and higher dose of TSG-6.

C57BL/6 mice were exposed to sham air or 3-day CS and injected i.v. with following treatment groups: 1) vehicle; 2) $3 \times 10^5$ hASC; 3) conditioned media from non-transfected hASC(ASC\textsuperscript{NT}), 4) & 5) from hASC transfected with TSG-6 siRNA (ASC\textsuperscript{TS}) with or without supplement of 0.2 µg recombinant human TSG-6 (rhTSG-6), or 6) from hASC transfected with control scramble siRNA (ASC\textsuperscript{SC}); 7) 0.2 µg rhTSG-6; 8) 2 µg rhTSG-6. Conditioned medium from ASC demonstrated equivalent effects as producer cells, which was compromised by loss of the key component TSG-6. Supplementing the lost amount of TSG-6 in conditioned medium with recombinant TSG-6 protein restored the efficacy of conditioned medium. The therapeutic effect of TSG-6 protein can be significantly enhanced (by ~10 fold) when synergized with other components in conditioned medium. Cell cycling (right) has a more sensitive response to treatment than absolute number of progenitors (left). Mean ± SEM, n=3/group. $^a$\textit{p}<0.05 (vs. AC); $^b$\textit{p}<0.05, (vs. vehicle).
ASC restored CS-induced cycling arrest of bone marrow progenitors

Slow or non-cycling state could be a key contributor to decreased clonogenic capacity of bone marrow progenitors observed above. To test this hypothesis, we assessed the cycling status of bone marrow progenitors from above treatment groups. Indeed, the percentage of immature progenitor cells in the S phase of the cell cycle (killed by tritiated thymidine in the assay) significantly decreased (CFU-GM by 75.8%, BFU-E by 90.5%, and CFU-GEMM by 93.2%) after 3-day CS but could be fully rescued by hASC, ASC-conditioned media, or excessive TSG-6 (Figure 34B). These results were consistent with previously observed trend of changes in progenitor numbers but with greater degree of difference among groups, suggesting cell cycling as a more sensitive indicator for outcomes of smoking and hASC treatment. In addition to clonogenic progenitors, ASC also enhanced cycling of primitive hematopoietic progenitors (Sca1+) by almost 3-fold (Figure 35).
Figure 35. Increased cycling of Sca1+ BM cells after hASC treatment.

Sca1+ bone marrow (BM) cells were sorted from mice exposed to air (AC) or 3-day cigarette smoking (CS) with or without injection of human ASC after Day 2. Cell cycling was evaluated by staining nuclei with propidium iodide and analyzing on flow cytometer.
4.4 Conclusion

Our results in Chapter 4 showed that cigarette smoke-derived toxic compounds caused acute cycling arrest and deficient clonogenicity of bone marrow hematopoietic progenitor cells (HPC), which progressed to depletion of phenotypic HPC after extended exposure. A single dose of $3 \times 10^5$ human ASC (i.v.) in midst of short-term exposure (3 days) and weekly repeated doses during chronic exposure (7 weeks) were both sufficient to reverse bone marrow progenitor deficiency caused by smoking. Notably the acute toxicity of smoke on bone marrow is also accompanied by upregulation of inflammatory cytokines such as TNF$\alpha$ and IL1$\beta$ in the lung, which triggered secretion of TSG-6, likely from ASC infused i.v. and trapped in the lung. Conditioned media from ASC as well as TSG-6 (though less effectively) antagonized myelosuppressive effects of chemical compounds in cigarette smoke extract in the \textit{in vitro} colony-forming-unit assay, suggesting potent antagonistic effects from trophic factors secreted by ASC, especially TSG-6. Our further knockdown experiments \textit{in vivo} showed that knockdown of TSG-6 in ASC or absence of TSG-6 in the ASC conditioned media results in almost complete loss of the therapeutic effect previously observed with regular ASC or conditioned media. Moreover, this lack of effect from ASC conditioned media can be compensated by supplementing the missing amount of TSG-6 protein, which by itself at much higher dose was sufficient to reverse the myelotoxicity of smoke \textit{in vivo}. 
4.5 Discussion

The widely distributed detrimental effects of cigarette smoking have previously been attributed to systemic inflammation, oxidative stress, endothelial injury, and hemodynamic dysfunctions. Little attention has been paid to the involvement of bone marrow progenitor cells in the pathogenesis of smoking-related diseases. Our results showed that the CS-induced damage in the bone marrow occurred rather acutely (1-3 days), well preceding the development of emphysema in the lung (typically 6 months), and certain types of progenitors (BFU-E and CFU-GEMM) recovered extremely slowly long after cessation of smoking. Given the importance of BM progenitor cells to peripheral tissue regeneration, it is highly likely that this early deficiency of maintenance cells subsequent to smoking contribute critically to the pathogenesis of numerous diseases related to cigarette smoking. To substantiate this hypothesis, it would be interesting to generate chimeric mice with bone marrow from smoking-resistant strain and evaluate their susceptibility to CS-induced emphysema. Notably, this early pathological changes were limited to the functionality of hematopoietic progenitors and could be easily overlooked if only phenotypes were examined. This discrepancy between functionality and phenotype was also noted in other occasions, supporting functional assays as a more sensitive method to detect BM abnormality.

For most experiments reported here, we used immune-competent mice treated with human ASC despite the concern of xenograft rejections, for the following considerations. 1) Cigarette smoking has a major impact on the immune system, which may affect other organ systems secondarily, which may increase its immunotolerance towards xenogeneic cell transplants; 2) mesenchymal stem cells lack HLA antigen and are immune-privileged; 3) more than 99% of xenograft MSC are rapidly removed by host environment within 48hrs after systemic administration; 4) human ASC have exhibited potent cross-species paracrine activities in other disease models; 5) findings from human ASC can be more easily translated into future clinical applications.
It has been increasingly recognized that the main therapeutic effect of intravenously delivered ASC originates from potent trophic factors released by the majority of cells trapped in the lung. That said, it is difficult to substitute hASC with their in vitro culture supernatant because secretome of hASC often adapt according to the interaction between hASC and their surrounding environment.\textsuperscript{162} A recent study has demonstrated that another type of mesenchymal stem cells, BMSC, were able to secrete anti-inflammatory cytokine TSG-6 when trapped in the lung and stimulated with TNFα.\textsuperscript{54} This in combination with our observation of an acute increase of inflammatory cytokine expressions in the lung after mice started smoking has prompted our interest in investigation of whether TSG-6 also plays a role in the ASC effect on CS-induced injury. Indeed, our data clearly showed that hASC not only respond to hTNFα but also to other inflammatory stimuli such as IL1β. Moreover this feedback mechanism extends to cross-species signals. Remarkably, we successfully substituted hASC with conditioned media generated based on this mechanism and established, for the first time, the importance of TSG-6 to the paracrine effect of hASC in the CS-induced BM hypoplasia model. These data laid a firm ground to the possibility of manufacturing more convenient off-the-shelf medical products to replace hASC at least for this indication so that the invasive liposuction procedure could be avoided.

TSG-6 belongs to the hyaluronan-binding protein family. Its potent anti-inflammatory effects have been demonstrated in multiple disease model and proposed mechanisms include binding to CD44 and interfering with NF-κb signaling pathway in macrophages to suppress TNFα secretion.\textsuperscript{47} Although pro-inflammatory cytokine such as TNFα could inhibit hematopoiesis,\textsuperscript{83} in our in vitro model of CS extract-induced progenitor suppression, the adverse effect came more likely from the direct toxicity of compounds in the cigarette smoke. And hASC, ASC-conditioned medium, and TSG-6 were all capable of antagonizing the toxicity, in accordance with they efficacy in vivo. This discovery of anti-myelosuppressive function of TSG-6 is unprecedented and may extend to other indications such as irradiation or chemotherapy-induced myelosuppression. That
said, the contribution of anti-inflammatory effect of TSG-6 to its overall benefit in the smoking model cannot be excluded, after all, we did observe a decline of TNFα transcripts in the lungs of mice treated with hASC. We also demonstrated, for the first time, that conditioned media from hASC if generated incorrectly (i.e. from hASC incapable of TSG-6 secretion) lack their expected efficacy. This may shed some light to previously failed attempts in other models to replace hASC with conditioned media. Remarkably, we were able to correct this flawed regime by supplementing the single missing component of TSG-6. It is worth pointing out though, despite the importance of TSG-6 to the effect of hASC, the efficacy of the cytokine alone is much lower than ASC-conditioned medium containing equivalent concentration of TSG-6. This indicates that there are other synergistic factors in the ASC-conditioned medium critical to the overall benefit but yet to be discovered.

Our observation of smoking-induced exhaustion of hematopoietic progenitor cells seemed paradoxical to previously observed smoking-associated leukocytosis in peripheral blood. On a closer look, they are not mutually exclusive since we did find an increased proliferation of hematopoietic cells in the bone marrow (Figure 36), which is not resolved by hASC or TSG-6. Although the number of LSK cells (Figure 28) and the cycling of Sca1+ cells (Figure 35) were not affected by short-term (3 days) smoking, the high demand of hematopoiesis (Figure 36) and arrested cycling of clononenic progenitors (Figure 34B) could lead to exhaustion of HSC (LSK) and primitive progenitor cell (Sca1+ cells) repository in the long run (Figure 28). In contrast, although ASC and TSG-6 did not suppress downstream hematopoiesis demand, they reactivated cycling of upstream clonogenic (CFU-GM, BFU-E, and CFU-GEMM) and primitive progenitors (Sca1+ cells), therefore avoided depletion of HSC (LSK cells).
Figure 36. Ki-67 staining of total BM cells.
Section slides of femurs from 3-day smoking mice were stained with rabbit anti-mouse Ki-67. Cells in brown color were Ki-67+ proliferating cells. Images showed distal end of the femur.
Chapter 5. Future Directions

5.1 Anti-inflammatory Aspect of ASC & TSG-6

Molecular Mechanisms

From a mechanistic standpoint of view, it would be of interest to further investigate target molecules in endothelial cells that are positioned downstream of TSG-6-mediated protection. Since CD44 is expressed abundantly on endothelial cells and has been confirmed to be a receptor for TSG-6 on macrophages, we plan to determine the expression and distribution of CD44 in endothelial cells before and after thrombin / TNFα / TSG-6 treatment. One previous study observed increased neutrophil transmigration subsequent to TNFα treatment and an inhibitory effect of TSG-6 link module. They excluded possible involvement of adhesion molecules in endothelial cells in this phenomenon by showing that TSG-6 link module did not change protein levels of the three key adhesion molecules ICAM-1, PECAM-1, and P-selectin in human bone marrow-derived endothelial cells. While this finding will have to be validated in our model, which uses TSG-6 whole protein and human umbilical vascular endothelial cells (HUVEC), we plan to also investigate potential changes in junction proteins (such as VE-cadherin) and the cell skeleton (such as F-actin), since they are more directly related to the regulation of inter-cellular gap formation and extravasation of neutrophils. If VE-cadherin is indeed affected, it would be interesting to further look into the VE-cadherin / β catenin signaling pathway, which has also been known to regulate permeability of HUVEC.

Applications in Disease Models

The protective effect on vascular barrier and inhibitory effect on lymphocyte proliferation we observed in vitro highly warrant further studies to test the efficacy of ASC and TSG-6 protein in relevant animal disease models. One example could be the abdominal aortic aneurysm (AAA). AAA is a highly insidious but fatal disease and comprises the 10th leading cause of death in men in the U.S. The only option for treatment is surgical repair, which is associated with significant morbidity, mortality, and expense. Vascular wall destruction
caused by MMP2 and MMP9 activation and excessive lymphocyte proliferation is the predominant event in the pathogenesis of AAA and the progression of the disease is directly related to inflammatory cytokine TNFα.\textsuperscript{164, 165} Our data showed that TSG-6 protected vascular barrier and inhibited lymphocyte proliferation. In combination with previous evidence that TSG-6 inhibits MMP2 and MMP9 activation as well as TNFα release from macrophage, they strongly imply ASC and TSG-6 as novel promising therapies for AAA.

5.2 Myeloprotective Aspect of ASC & TSG-6
Effects of Smoking on HSC

Our data from colony-forming-unit assays demonstrated an acute toxicity of CS on all 3 lineages of HPC, namely CFU-GM, BFU-E, and CFU-GEMM. Furthermore, the numeric change of Lin-Sca1+c-Kit+ cells (enriched for HSPC) suggested a possibility that with sufficient exposure, the toxicity of CS may also extend to HSC. To pursue this hypothesis, it is helpful to differentiate effects of CS on long-term HSC (CD34\textsuperscript{-}CD135\textsuperscript{-} LSK), short-term HSC (CD34\textsuperscript{+}CD135\textsuperscript{-} LSK), and MPP (CD34\textsuperscript{+}CD135\textsuperscript{+} LSK) within the LSK cell population. If phenotypic short or long-term HSC were indeed reduced by CS, competitive repopulation assays could be used to further verify potential damages on functional HSC. Specifically, BM HSC from C57BL/6 mice (CD45.2\textsuperscript{+}) treated with air, CS, or CS + ASC will be co-transplanted with BM HSC from BoyJ (CD45.1\textsuperscript{+}) mice into recipient B6/BJ-F1 mice (CD45.1\textsuperscript{+} CD45.2\textsuperscript{+}). Influence of CS and ASC on HSC will be determined by comparing engraftment of CD45.2\textsuperscript{+} cells in recipient mice 1, 2, 4, and 6 months after transplantation.

Molecular Mechanisms

To further dissect the molecular effectors for smoke / TSG-6, we plan to compare gene signatures of HPC among three treatment groups: air control, cigarette smoke, and cigarette smoke + ASC/TSG-6 and look for genes changed after smoke and reversed by ASC/TSG-6 treatment. The quantitative changes in LSK cells (enriched for hematopoietic stem and progenitor cells) from mice
exposed to 7-week CS suggest that phenotypic changes may become evident with long-term CS exposure, which allows for targeted analysis of different HPC populations, such as CMP, CLP, GMP, MPP, etc. Therefore, after changes in these phenotypic progenitors were confirmed, they could then be selected by flow cytometry and analyzed for regulatory genes by PCR arrays. High throughput PCR array will be necessary given the scarcity of LSK cells in the bone marrow. Information from high throughput arrays can then be verified by qPCR and Western blot to further narrow down the targets. We will then be able to design gain / loss-of-function experiments to modulate these factors and evaluate their impact at the cellular level.

Effects of Smoking on Bone Marrow Niche Environment

The toxicity of cigarette smoking may not only be restricted to bone marrow hematopoietic progenitor cells per se, but also extends to the entire niche environment of bone marrow. After all, studies have shown that smoking history is related to worse prognosis after bone marrow transplantation, which supports the notion that chronic smoke exposure may result in a harsh receptive bone marrow environment. If the bone marrow environment did suffer from smoking-induced malfunction, then in addition to cycling and clonogenicity, engraftment and mobilization of hematopoietic stem and progenitor cells (HSC) may also be affected. The engraftment abnormality could be studied by transplantation of HSC from regular mice to recipient mice with or without previous exposure to smoking. And the mobilization dysfunction could be verified by comparing mobilized HSC in peripheral blood in mice exposed to smoking or ambient air. Deficient HSC mobilization, if verified, would shed some light into the rationale behind the correlation between smoking history and slower wound healing and worse prognosis of cardiovascular diseases.

Hematological Applications of ASC and TSG-6

Attempts to enhance engraftment of HSC with ASC have been made only in recent two years whereas TSG-6 has never been applied to hematological
indications. The remarkable protective effects of both in our model of smoking-induced myelosuppression highly warrant further efforts to study them in the context of myelosuppression from other causes, such as irradiation and chemotherapy. After all, many etiological factors responsible for myelosuppression such as oxidative stress and inflammation are shared by smoking and irradiation / chemotherapy. Besides, the quick recovery of cell cycling as a result of ASC / TSG-6 treatment could contribute to the recovery of diminished viable stem / progenitor cell repository from harsh myelotoxins. Taken together with our finding of the strong potency of TSG-6 against lymphocyte proliferation in Chapter 2, it suggests TSG-6 may become a promising novel therapy for GVHD based on its function to both promote HSC recovery and suppress immune rejection, especially for allogeneic transplants.
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Curriculum Vitae

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Education/Training

2008-2012 Ph.D.,
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Memberships

2009-Present American Physiological Society
2010-Present International Federation for Adipose Therapeutics and Science
2011-Present Indiana Physiological Society
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Professional Service

2011 Reviewer for Journal of Leukocyte Biology

Teaching

2011 Mentor for rotation Senior Undergraduate Student (Krista Podell, University of Indianapolis, IN)

2011 Mentor for Biomedical Engineering Senior Design Group (Alisa Beal, etc. Purdue University, IN)
2010 Mentor for rotation Medical Student (Jennifer Bell, Lake Erie College of Osteopathic Medicine, FL)

**Assisted Grant Writing**

**2012**  
Applicant: Michael P. Murphy, M.D.; Keith L. March, M.D., Ph.D.  
Title: Attenuation of Abdominal aortic aneurysm with mesenchymal stem cells  
Funding: R01, National Institute of Health  
Outcome: Unknown

**2012**  
Applicant: Keith L. March, M.D., Ph.D.  
Title: Restoration of Bone Marrow Hematopoietic Function Impaired by Cigarette Smoking: Novel Therapy Based on Adipose Stromal Cells and the Secreted Factor TSG6  
Funding: Bone Marrow Failure Research Program Idea Award, Department of Defense  
Outcome: Unfunded

**2011**  
Applicant: Keith L. March, M.D., Ph.D.  
Title: Impact of Cigarette Smoking on Mobilization of Bone Marrow Stem and Progenitor Cells and Possible Treatment  
Funding: Hypothesis Development Award, Department of Defense  
Outcome: Unfunded

**2011**  
Applicant: Irina Petrache, M.D.; Keith L. March, M.D., Ph.D.  
Title: Direct and Bone-Marrow Mediated Effects of Adipose Stem Cells in Emphysema  
Funding: R01, National Institute of Health  
Outcome: Funded
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<td>2011</td>
<td>Keith L. March, M.D., Ph.D.</td>
<td>Adipose-derived Stem Cells Inexpensively Produce a Cocktail of Novel and Broad-Spectrum Anti-pathogenic Agents</td>
<td>Grand Challenges Explorations, Bill &amp; Melinda Gates Foundation</td>
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<td>2011</td>
<td>Rajashekhar Gangaraju, Ph.D.; Keith L. March, M.D., Ph.D.</td>
<td>Neuronal Repair and Vascular Stabilization with Adipose Stromal Cells</td>
<td>Young Investigator Grants, Alcon Research Institute</td>
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<td>2009</td>
<td>Keith L. March, M.D., Ph.D.</td>
<td>Vasculogenic Potential of Adipose Stromal Cells: Provision of Tissue Perfusion and Islet Survival in Health, Aging, and Diabetes</td>
<td>VA Merit Review Grant, Department of Veterans Affairs</td>
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<td>2009</td>
<td>Keith L. March, M.D., Ph.D.</td>
<td>Adipose Stromal Cells as a Novel Therapy for Ionizing Radiation Toxicity: Evaluation in Murine Radiation-induced Bone Marrow Aplasia</td>
<td>Research Support Funds Grant, IUPUI Office of the Vice Chancellor for Research</td>
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Grant Support

2010-2012  PI: Jie Xie, M.D.  Mentor: Keith L. March, M.D., Ph.D.

Title  Cigarette Smoking effects on Marrow Stromal Cells and Angiogenesis: Modulation by Adipose Stromal Cell Transplantation

Funding  Predoctoral Fellowship (11PRE5730005)  American Heart Association Midwest Affiliation

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2012  The Kenneth L. Barker Young Investigator Award  Society for Experimental Biology and Medicine

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Conference Presentations

1. **Podium Presentation**: Correcting Smoke-Induced Bone Marrow Dysfunction: Moving from Adipose-Derived Stem Cells to Their Secretome. **Jie Xie**, Dongni Feng, Todd G. Cook, Mary J. Van Demark, Kelly S. Schweitzer, Brian H. Johnstone, Irina Petrache, Hal E. Broxmeyer, Keith L. March. 10th Annual Symposium on Adipose Stem Cells and Clinical Applications of Adipose Tissue; Quebec, Canada, 2012.
2. **Poster:** *Human adipose-derived stem cells attenuate cigarette smoke induced bone marrow hypoplasia via secretion of anti-inflammatory cytokine TSG6.*


4. **Podium Presentation:** *Human Adipose-Derived Stem Cells Protect Against Cigarette-Smoke Induced Bone Marrow Hypoplasia through Paracrine Factors.* **Jie Xie**, Kelly S. Schweitzer, Brian H. Johnstone, Todd G. Cook, Dongni Feng, Marjorie E. Albrecht, Yong Gao, Matthew J. Justice, Scott H. Cooper, Hal E. Broxmeyer, Irina Petrache, Keith L. March. 9th Annual Symposium on Adipose Stem Cells and Clinical Applications of Adipose Tissue; Miami, 2011.

5. **Abstract:** *Protection of mouse pancreatic islet viability from hypoxia induced injury by adipose derived-stromal cells conditioned media.* Dongni Feng, **Jie Xie**, Brian H. Johnstone, Dmitry O. Traktuev, Carmella Evans-Molina, Keith L. March. 9th Annual Symposium on Adipose Stem Cells and Clinical Applications of Adipose Tissue; Miami, 2011.

Patent
Jie Xie and Keith L. March. Protection Against Chemical-Induced or Other (i.e., Endogenous) Myelosuppression Based on the Protein TSG6, Adipose Stromal Cells (ASC), or ASC Conditioned Medium. April, 2012 (IURTC 12171)

Publications