



Dissection of the Human Multipotent Adult Progenitor Cell Secretome by Proteomic Analysis

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ABSTRACT

Multipotent adult progenitor cells (MAPCs) are adult adherent stromal stem cells currently being assessed in acute graft versus host disease clinical trials with demonstrated immunomodulatory capabilities and the potential to ameliorate detrimental autoimmune and inflammation-related processes. Our previous studies documented that MAPCs secrete factors that play a role in regulating T-cell activity. Here we expand our studies using a proteomics approach to characterize and quantify MAPC secretome components secreted over 72 hours in vitro under steady-state conditions and in the presence of the inflammatory triggers interferon- γ and lipopolysaccharide, or a tolerogenic CD74 ligand, RTL1000. MAPCs differentially responded to each of the tested stimuli, secreting molecules that regulate the biological activity of the extracellular matrix (ECM), including proteins that make up the ECM itself, proteins that regulate its construction/deconstruction, and proteins that serve to attach and detach growth factors from ECM components for redistribution upon appropriate stimulation. MAPCs secreted a wide array of proteases, some detectable in their zymogen forms. MAPCs also secreted protease inhibitors that would regulate protease activity. MAPCs secreted chemokines and cytokines that could provide molecular guidance cues to various cell types, including neutrophils, macrophages, and T cells. In addition, MAPCs secreted factors involved in maintenance of a homeostatic environment, regulating such diverse programs as innate immunity, angiogenesis/angiostasis, targeted delivery of growth factors, and the matrix-metalloprotease cascade. *STEM CELLS TRANSLATIONAL MEDICINE 2013;2:745–757*

INTRODUCTION

There is accelerating interest in developing adult-derived mesenchymal stromal and other stem cells as therapeutic agents for the treatment of graft versus host disease (GVHD), cardiac indications, central nervous system (CNS) injury, and CNS disease pathologies [1–5]. Adult stromal cells have been isolated from multiple tissues and characterized [6]. Adult-derived adherent stromal cells demonstrate a number of favorable characteristics, including genetic stability, extensive expansion capacity, and low immunogenicity profiles [7]. These properties could prove invaluable in an allogeneic hematopoietic stem cell transplantation setting in which the patient receives bone marrow or blood stem cells from a closely but non-genotypically identical human leukocyte antigen (HLA)-matched donor [4, 8, 9]. Functional improvement after administration of adult-derived cells has been demonstrated in multiple preclinical models of injury or disease, but the mechanisms by which these outcomes are accomplished remain poorly

understood [9, 10]. An understanding of the specific mechanisms by which beneficial effects are obtained after treatment with adult stem cells will be important for refining both dosage and route of delivery of these cells as cellular therapeutics continue to expand into clinical development.

Multipotent adult progenitor cells (MAPCs) (MultiStem; Athersys, Inc., Cleveland, OH, <http://www.athersys.com>) were originally described as a subpopulation of adherent stem cells that could be isolated from adult bone marrow and other adult tissues [11]. The bone marrow compartment contains populations of adult stromal cells that exist in a discernible hierarchy [12]. More primitive cells such as MAPCs or marrow-isolated adult multilineage inducible cells [13] and less primitive stem cells such as mesenchymal stem cells (MSCs) all copurify, and clonal populations are obtained by manipulating culture conditions [14].

Both MAPCs and MSCs have been shown to have strong immunomodulatory properties, and we have recently shown that rodent and human

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cell populations from bone marrow that were expanded at large scale via conditions specified for MAPC isolation are safe and can positively modulate the immune system [15, 16]. Based on these properties MAPCs have been “fast-tracked” into clinical trials. However, a detailed molecular understanding of the basic biology of these cells at rest and under conditions of variable stimuli remains absent and elusive. The studies described in this article were conducted to identify immunomodulatory factors secreted by MAPCs and to determine the effect of potential licensing or triggering agents on secretion of these factors. This information will be used to guide the design of future in vitro interaction studies between human MAPCs and peripheral blood mononuclear cells and in vivo experiments using a rodent model of GVHD evaluating the requirement, hierarchical importance, and redundancy of the various factors identified.

A straightforward proteomics approach involving liquid chromatography-coupled tandem mass spectrometry (LC-MS/MS) detection and bioinformatics was used to take an inventory and categorize the array of factors secreted by MAPCs. We sought to obtain a global overview of the immunomodulatory potential of MAPCs by characterizing the manufacturing capacity and secreted protein profiles of these cells over a 72-hour period under standard culture conditions in the absence of any exogenous stimuli, and then monitored changes in the secreted molecular constituents following treatment of MAPCs with inflammatory stimuli interferon (IFN)- γ and lipopolysaccharide (LPS), or a tolerogenic stimuli, RTL1000, a CD74 ligand [17–20]. A recent review by Hynes and Naba [21] provides an inventory of the molecular constituents and functions of the extracellular matrix (ECM), and the results presented here document that many of the known ECM regulatory components are directly manufactured by MAPCs, which secrete molecules that control angiogenesis, the matrix metalloproteinase (MMP) cascade, and effectors of both innate and adaptive immunity. In addition to classic secreted molecules, the MAPC secretome contains (a) premanufactured molecules that are stored in secretory granules and rapidly released from cells in response to external stimuli, and (b) cell-surface molecules proteolytically shed by ectodomain shedding. The results presented in this report reveal an array of factors known to be involved in regulating and integrating aspects of inflammation and angiogenesis, including the trafficking of leukocytes to sites of inflammation, and are discussed in the context of the potential utility of these cells in prevention and treatment of inflammatory disorders.

MATERIALS AND METHODS

Chemicals and Reagents

IFN- γ was purchased from R&D Systems (Minneapolis, MN, <http://www.rndsystems.com>; catalog no. 2851F100), and LPS was from Sigma-Aldrich (St. Louis, MO, <http://www.sigmaaldrich.com>; catalog no. L4391).

RTL1000

We have previously described recombinant T cell receptor ligands (RTLs) derived from the β -1 and α -1 domains of major histocompatibility (MHC) class II molecules bearing covalently tethered antigens [22]. These molecules effectively tolerize Ag-specific CD4⁺ T cells [23] and modulate the inflammatory potential of CD11b⁺ monocytes [20]. Cloning, expression, and purifi-

cation of the RTL protein constructs from inclusion bodies produced in *Escherichia coli* have been previously described [24]. Characterization and batch-to-batch quality control validation for RTLs [25] have also been described extensively in previous publications [22, 26]. The RTL1000 used in this study was recently successfully used in a phase I clinical trial for treatment of multiple sclerosis [27]. It is extremely stable as assessed by biochemical and biophysical integrity and biological activity (inhibiting clinical signs of experimental autoimmune encephalomyelitis in DR2-Tg mice) over a 42-month follow-up period.

Human MAPC Cultures and MAPC-Conditioned Media

MAPCs meet the formal criteria for designation of cells as MSCs, a prototype for adherent stem and progenitor cells, defined in a position statement of the International Society for Cellular Therapy [28]. This designation requires that the in vitro expanded cells be plastic-adherent and express cell-surface molecules, including CD73, CD90, and CD105, in the absence of hematopoietic markers, including CD14, CD34, CD45, and HLA-DR. MAPCs can be distinguished from MSCs on the basis of cellular phenotype, size, transcriptional profile, and expansion capacity. Compared with standard MSC culture conditions, MAPCs are isolated using hypoxic conditions in media supplemented with growth factors epidermal growth factor and platelet-derived growth factor and kept at subconfluent culture density [6]. MAPCs can be expanded under defined low-serum conditions for more than 100 population doublings without telomere shortening and remain karyotypically normal [11, 29]. Clinical-grade MAPCs (MultiStem) were manufactured by Athersys. Human MAPC cultures were derived from bone marrow aspirates donated by human volunteers (lots 061269, BMC135, and BMC167), and bone marrow was processed as previously described [30]. In brief, human MAPCs were isolated from bone marrow aspirate, obtained with consent from a healthy donor, and cultured in fibronectin-coated plastic tissue culture flasks. Cell cultures were maintained under low oxygen tension in a humidified 5% CO₂ atmosphere. Cells were cultured to subconfluence in MultiStem culture medium consisting of low-glucose Dulbecco's modified Eagle's medium (Life Technologies/Invitrogen, Grand Island, NY, <http://www.lifetech.com>) supplemented with fetal bovine serum (Atlas Biologicals, Fort Collins, CO, <http://www.atlasbio.com>), insulin, transferrin, and selenium liquid medium supplement (Sigma-Aldrich), MCDB (Sigma-Aldrich), platelet-derived growth factor (R&D Systems), epidermal growth factor (R&D Systems), dexamethasone (Sigma-Aldrich), penicillin/streptomycin (Life Technologies), 2-phospho-L-ascorbic acid (Sigma-Aldrich), and linoleic acid-albumin (Sigma-Aldrich). Cells were passaged every 3–4 days and harvested using trypsin/EDTA (Life Technologies). Flow cytometric analysis of surface-expressed antigens confirmed the homogeneity of the MAPCs used in this study and confirmed that they met previously described release criteria [6], including staining positive (>90%) for CD49c and CD90 and negative (<5%) for MHC class II and CD45 (antibodies used were from BD Biosciences, San Jose, CA, <http://wwwbdbiosciences.com>). Cells were cryopreserved in Plasma-Lyte A (Baxter, Deerfield, IL, <http://www.baxter.com>) with dimethyl sulfoxide and human serum albumin. Immediately prior to their use MAPCs were thawed, washed twice with xeno-free medium containing 1% human serum, and then cultured for a minimum of three passages. MAPCs were then treated with various stimuli, including

IFN- γ (2 ng/ml), LPS (10 μ g/ml), and RTL1000 (5 μ g/ml). Conditioned medium (CM) was collected at 24 hours (15% confluent), 48 hours (30% confluent), and 72 hours (60% confluent) in 50-ml conical tubes (BD Biosciences). The conditioned medium samples were spun down at 400g for 5 minutes at 4°C to remove cells and debris, and the supernatants were transferred to new 50-ml conical tubes. MAPC-conditioned medium (MAPC-CM) samples were concentrated 50-fold with an Amicon Microcon Ultracel YM-3 3000 molecular weight cutoff centrifugal filter (Millipore, Billerica, MA, <http://www.millipore.com>), snap-frozen on dry ice, and stored at -80°C until analysis.

Processing of Media and MAPC-CM Samples and Immunodepletion of Major Serum Components From MAPC-CM for Secretome Analysis

Samples were thawed and assayed for protein content using a bicinchoninic acid (BCA) assay and bovine serum albumin standard (Pierce, Rockford, IL, <http://www.piercenet.com>) [31]. MAPC-CM samples were buffer exchanged into Agilent buffer A (Agilent Technologies, Santa Clara, CA, <http://www.agilent.com>; proprietary medium formulation) and concentrated, and the total amount of protein present in the samples was determined (BCA) (Pierce). Recognizing that the presence of even 1% serum limits the depth of coverage and identification of secreted cell products, MAPC-CM samples were immunodepleted using a serum immunodepletion column (Agilent multiple affinity removal system [MARS-14]) of the 14 most common serum proteins that make up 98% of the total protein in serum prior to characterization by LC-MS/MS. The immunoaffinity column antibodies and buffer components are designed to interact with the major serum components in a denaturing but nonreducing buffer A, resulting in the removal of the major serum components without removing secretome components that potentially bind to these major serum proteins (Virginia Curtiss, Ph.D., Agilent Technologies, personal communication).

Trypsinization of Secretome Samples

Serum-depleted medium and MAPC-CM samples were concentrated and assayed for protein content by BCA assay. Ten micrograms total protein per sample was treated with trypsin at 37°C overnight at a ratio of enzyme/substrate of 1:25, and the reaction was stopped by the addition of formic acid as previously described [32].

Mass Spectrometry Analysis of MAPC Secretome Peptides

Forty microliters (10 μ g of total medium or MAPC-CM protein digested with trypsin) of each sample was separated by reverse phase liquid chromatography while collecting data-dependent MS/MS spectra on the eluted peptides. Peptides were separated using an Agilent 1100 series capillary LC system and an LTQ Velos linear ion trap mass spectrometer (ThermoFisher, San Jose, CA, <http://www.thermofisher.com>). Electrospray ionization was performed with an ion max source fitted with a 34 gauge metal needle and 2.4 kV potential. Samples were applied at 20 μ l/minute to a trap cartridge (Michrom BioResources, Inc., Auburn, CA, <http://www.michrom.com>) and then switched onto a 0.5 \times 250 mm Zorbax SB-C18 column with 5 μ m particles (Agilent Technologies) using a mobile phase containing 0.1% formic acid, 7%–30% acetonitrile gradient over 220 minutes, and 10 μ l/minute flow rate. Data-dependent collection of MS/MS spectra used

the dynamic exclusion feature of the instrument control software (repeat count equal to 1, exclusion list size of 10, exclusion duration of 30 seconds, and exclusion mass width of -1 to +4) to obtain MS/MS spectra of the five most abundant parent ions (minimum signal threshold of 1×10^4) following each survey scan from m/z 350 to 2,000. The tune file was configured with no averaging of microscans, a maximum inject time of 200 ms, and automatic gain control targets of 3×10^4 in mass spectrometry mode and 1×10^4 in multiple-stage mass spectrometry mode.

Data Analysis

An in-house script was used to create DTA-format files from the RAW files using extract_msn.exe (version 5.0, ThermoFisher) with a molecular weight range of 550–4,000, an absolute threshold of 500, group scan setting of 1, and a minimum of 25 ions. A human species subset of the Sprot (version 2011.06) protein database (20,235 proteins) was prepared with concatenated reversed entries (and common contaminants) using scripts available at the Proteomic Analysis Workbench (<http://www.ProteomicAnalysisWorkbench.com>) and searched with SEQUEST (version 28, revision 12; ThermoFisher). Parent ion and fragment ion tolerances of 2.5 and 1.0 Da were used with calculated average and monoisotopic masses, respectively. Cysteine had a static modification mass of +57 Da, and trypsin specificity was used. An in-house suite of programs [33] was used to provide a Peptide Prophet-like discriminant function [34] scoring to identify “correct” peptides and discard “incorrect” peptides using sequence-reversed matches to estimate the false discovery rate (FDR). Of the 798,803 acquired MS2 scans from the 24 samples, there were 132,333 that passed score thresholds with 6,649 matches to reversed sequences, giving an estimated overall peptide FDR of 5.3%. Protein identification lists were prepared using standard parsimony principles. Proteins were required to have two or more fully tryptic peptides with distinct sequences. Different charge states of the same peptide sequence were not considered as unique peptides. The number of identified proteins per sample ranged from 59 to 287 (nonredundant counts, excluding contaminants), and the protein FDR per sample ranged from 1.2% to 6.4%. An in-house algorithm was used to compare identified peptide sets between proteins having peptides in common and group together highly homologous identifications into families. Shared and unique peptide status was recomputed across all proteins after family grouping. Shared peptide counts were fractionally split on the basis of relative total unique counts of the proteins that had those peptides in common. These corrected total spectral counts per protein were used in the quantitative comparisons described below. There are a number of factors that influence the number of spectral counts for a particular protein. For example, a protein would be under-represented or even undetected if, by virtue of its structure or association with other elements, it was not efficiently cleaved by trypsin. Similarly, certain peptides, such as ones shorter than 6 or longer than 20 residues, produce MS/MS spectra that are difficult to match to peptide sequences, and therefore, these peptides would be under-represented. One parameter that strongly affects the count of peptides in the analysis is the size of the parent protein. At equimolar concentrations, a large protein will generate more peptides than a small one of similar composition and structure. To normalize for this effect, we divided the spectral count for a given protein by the molecular mass of that protein so the abundance more closely resembled molar concentrations rather than

Table 1. MAPC protein secretome: Functionally distinct classes of molecules identified by HPLC-MS/MS

Protein name (ID)	Description, functional annotation, and references	Spectral count Δ		
		IFN- γ	LPS	RTL1000
A. ECM components/cell adhesion				
1. Collagens, collagen-binding proteins involved in adhesion/cell attachment/cell junctions/cell migration				
Type I (CO1A1, A2)	Fibrillar collagen [21].	nc, \uparrow	nc, \uparrow	nc, nc
Type VI (CO6A1, A2, A3)	Basement membrane collagen (cell-binding) [21].	\uparrow , \uparrow , nc	\uparrow , \uparrow , nc	\uparrow , \uparrow , \uparrow
Fibrilin-1 (FBN1)	Homotypic binding to form threadlike microfibrils that form larger-order elastic fibers. Binds to LTBP-1, -2, and -4 [54].	nc	\uparrow	nc
Biglycan (PGS1)	Binds BIGH3 [55].	\uparrow	\uparrow	\uparrow
Decorin (PGS2)	Binds BIGH3 [55].	\downarrow	nc	nc
BIGH3 (BGH3)	Synonyms: TGF- β -induced collagen binding protein, keratoepithelin, TGFBI. Binds collagen VI, decorin and biglycan [55]. Regulates MMP14 levels and collagen turnover [56].	nc	nc	nc
Periostin (POSTN)	High homology to BGH3 [57]; binds type I collagen, $\alpha5\beta3$, $\alpha5\beta5$, and $\alpha6\beta4$ integrins [58]. Expression regulated by IL-13 [59, 60]. Periostin increases the level of active TGF- β [60], driving CD4+CD25-FOX-P3+, T-reg phenotype [61].	nc	\uparrow	nc
Nidogen-2, (NID2)	Bind laminin, collagen IV, and perlecan [62, 63].	nc	nc	nc
Laminin (LAMB1)	Laminin chain B; bind $\alpha3\beta1$, $\alpha6\beta1$, and $\alpha6\beta4$ integrins found on lung epithelium [58].	nc	\uparrow	nc
Versican (CSPG2)	Mediates cell-ECM interaction. Enhances cell attachment and the expression of $\beta1$ integrins and fibronectin. Protects cells from oxidative stress-induced apoptosis [64]. Regulator of inflammation [65].	nc	nc	nc
Testican-1 (TICN1)	Regulates degradation of ECM [66]; inhibits activation of pro-MMP2 by MMP14 [67]; inhibits cathepsin L [68].	\uparrow	\uparrow	\uparrow
Cadherin-2 (CADH2)	Calcium-dependent homophilic cell adhesion protein.	nc	nc	nc
Calsyntenin-1 (CSTN1)	Also termed alcadein. Single-pass type I TM protein constitutively cleaved by ADAM10 or ADAM17 [69]. Cleavage supports proper vesicle trafficking [70].	nc	\uparrow	nc
Osteopontin (OSTP)	Multifunctional protein involved in bone resorption, immune cell activation, inhibition of vascular calcification, and ECM remodeling [71–75].	\downarrow	\downarrow	nc
2. Proteases and protease regulators				
MMP1	Collagen degradation, ECM remodeling [76].	nc	nc	nc
MMP2	ECM remodeling [76].	\uparrow	\uparrow	nc
Cathepsin B (CATB)	ECM remodeling [76].	nc	nc	nc
Serglycin (SRGN)	Regulates MMP zymogen activation [77, 78]. Promotes storage of proteases and chemokines [79].	\downarrow	\uparrow	nc
Kallistatin (KAIN)	Tissue kallikrein inhibitor.	nc	nc	\downarrow
TIMP1	Complexes with metalloproteinases (such as collagenases) and irreversibly inactivates them by binding to their catalytic zinc cofactor. Also mediates erythropoiesis in vitro. Acts on MMP1, 2, 3, 7, 8, 9, 10, 12, 13, and 16. Does not act on MMP14, MMP19, or ADAM-17 [80, 81]. Antiapoptotic and cell signaling activity separate from metalloproteinase activity [82].	nc	nc	\uparrow
TIMP2	Specific inhibitor of MMP2 [83]. Antiapoptotic and cell signaling activity separate from metalloproteinase activity [82].	nc	nc	nc
Serpin E1 (PAI-1)	Serine protease inhibitor also termed PAI-1 [84]. PAI-1 binds LDL-receptor-related protein, mediating attachment, detachment, and migration of cells [85]. Binds vitronectin, regulates CD4 levels [86]. Regulates angiogenesis/angiostasis. Inactivates both urokinase- and tissue-type plasminogen activator, forming 1:1 complexes with dissociation constants in the pM range [87]. Activated by negatively charged phospholipids [88].	nc	nc	nc
Serpin E2 (GDN)	Serine protease inhibitor (also termed glia-derived Nexin) with activity toward thrombin, trypsin, and urokinase.	\downarrow	nc	nc
Pro-collagen C-endopeptidase enhancer-1 (PCOC1)	Also termed BMP1 enhancer protein, PCPE. Binds to the C-terminal pro-peptide of type I pro-collagen and enhances BMP1 (pro-collagen C-proteinase) activity \sim 20-fold [89, 90]. C-terminal processed portion of PCPE (CT-PCPE) inhibits MMP-2 IC ₅₀ \sim 560 nM [91]. The net result of both activities is facilitating increased collagen deposition.	\uparrow	\uparrow	nc

Table 1. (Cont'd)

Protein name (ID)	Description, functional annotation, and references	Spectral count Δ		
		IFN- γ	LPS	RTL1000
B. Growth factors/modulators, wound healing Secreted protein acidic and rich in cysteine (SPRC)	Regulates cell growth through interactions with the ECM and cytokines. Binds calcium and copper, several types of collagen, albumin, thrombospondin, endoglin [92], PDGF, and cell membranes. Inhibits endoglin-dependent TGF- β activity [93]; modulates MMP activity [94]; mediates ECM remodeling [95]; regulates cathepsin B-mediated cell infiltration via collagen I and α 2 β 1 integrin axis [96].	nc	nc	nc
1. Inhibitors of mature TGF- β Follistatin-related protein-1, -3 (FSTL1, 3)	TGF- β and BMP antagonists; evoke innate immune response through CD14 and TLR4, inducing IL-6 secretion [97]. Endogenous TLR4 agonist; suppresses production of MMPs and prostaglandins [98].	\uparrow , nc	\uparrow , \uparrow	\uparrow , nc
Vasorin (VASN)	TGF- β /BMP signaling [99, 100].	\uparrow	\uparrow	\uparrow
2. Regulators of latent TGF- β Latent TGF- β -binding protein-1 (LTBP1) Thrombospondin-1, -2 (TSP1, 2)	TGF- β /BMP signaling [101]. Adhesive glycoproteins that mediate cell-to-cell and cell-to-matrix interactions. Bind heparin. Ligand for CD36 mediating antiangiogenic properties. Involved in TGF- β regulation. MMP-2 substrate [76]. TSP2 transcription is downregulated in MAPCs after exposure to soluble factors secreted by α CD3/ α CD28-activated PBMCs [100].	nc, \downarrow	\uparrow , nc	nc, nc
Angiotensin-related protein 4 (ANGL4)	Monomer regulates angiogenesis and inhibits proliferation, migration, and tubule formation of endothelial cells. Regulates glucose homeostasis, lipid metabolism, and insulin sensitivity. In response to hypoxia, homo-oligomerizes and interacts with the ECM to maintain a dynamic reservoir of the protein [102].	\uparrow	\uparrow	nc
Galectin-1 (LEG1)	Negative regulator of T cells and immunosuppressive agents [103]. LEG1 is an MMP-2 substrate [76, 104–106].	nc	\downarrow	\downarrow
Connective tissue growth factor (CTGF)	Associated with wound healing. Binds integrins [107], growth factors including VEGF [108], and ECM proteins fibronectin, perlecan, and fibulin-1 [109].	\downarrow	nc	nc
Insulin-like growth factor binding protein-4, -5, and -7 (IBP4, IBP5, IBP7)	Binds LTBP-1 [110].	\uparrow , nc, nc	\uparrow , \uparrow , nc	\uparrow , nc, nc
Vimentin (VIME)	Mesenchymal marker; upregulated by periostin [111]. Bacterial killing [112]. MMP14 membrane translocation [113]. Involved in clearing cell debris by binding <i>N</i> -acetylglucosamine (GlcNAc) [114, 115]. Targeted cell-surface activation of LTG- β .	nc	nc	\downarrow
Semaphorin-7A (SEM7A)	Negative regulator of T cells [116].	nc	nc	\uparrow
α -Enolase (ENOA)	Metalloenzyme involved in the last step of glycolysis.	\uparrow	nc	nc
CD166 (ALCAM)	Membrane-bound form modulates cell-cell interactions within tissue niches [117]. Ectodomain cleavage by ADAM-10 and ADAM-17 [118]. Controls MMP activity [119].	\downarrow	\uparrow	\uparrow
C. Innate immune system components (early protection from pathogens) Pentraxin-2, -3 (SAMP, PTX3)	Pentraxins are induced by IL-1 β and TNF. Facilitate pathogen recognition by macrophages and DCs. SAMP is a serum amyloid P component [120].	nc, nc	nc, \uparrow	nc, nc
(IGHG-1, IGHD)	Recognition of bacteria, viruses, cell waste/modified self [121].	\downarrow , \downarrow , nc	\downarrow , nc	\downarrow , nc
Calgranulin-A, S10A8	Ca ²⁺ binding [122]. Broad-spectrum antimicrobial activity towards bacteria and fungi [123].	\uparrow	nc	nc
Lactotransferrin (TRFL)	Antimicrobial, antiviral, and antiparasitic activity that depends on the extracellular cation concentration [124].	nc	nc	\uparrow
D. Immune modulation (ICAM1)	sICAM-1 inhibits neutrophil and macrophage recruitment during inflammation. Binds β 2 integrins on leukocytes and decrease their availability for cell-cell interactions [118, 125].	\uparrow , \uparrow	nc	\uparrow
E. Cytokines/chemokines CXCL1 and 3 (GROA; CXCL3)	Cell communication and trafficking [126–128].	nc, nc	\uparrow , \uparrow	nc, nc

A >2-fold difference at 24 hours of treatment was criterion for no change, increase (\uparrow), or decrease (\downarrow) as indicated.

Abbreviations: BMP, bone morphogenetic protein; ECM, extracellular matrix; HPLC-MS/MS, high-performance liquid chromatography tandem mass spectrometry; ICAM, intercellular adhesion molecule; IFN, interferon; IL, interleukin; LDL, low-density lipoprotein; LPS, lipopolysaccharide; MAPC, multipotent adult progenitor cell; nc, no change; PAI, plasminogen activator inhibitor; PBMC, peripheral blood mononuclear cell; PDGF, platelet-derived growth factor; TGF, transforming growth factor; TNF, tumor necrosis factor.

weight concentrations. We recognize that the relationships may not be strictly linear and accordingly use the term “apparent abundance” when comparing the signals from two proteins. The list of all proteins identified, their normalized spectral counts, and annotations are found in supplemental online Table 1.

Bioinformatics

Swiss protein identifiers (more human-informative but less stable identifiers) were mapped to their Swiss-Prot accession numbers for subsequent analysis when needed. The potential for MAPCs to manufacture the secretome components identified by LC-MS/MS was confirmed by microarray analysis. Samples were run in duplicate on Human WG6 V2 gene chips (Illumina platform; Illumina Inc., San Diego, CA, <http://www.illumina.com>), and the data were analyzed using Illumina Bead Studio software, Excel (Microsoft, Redmond, WA, <http://www.microsoft.com>), and Invitrogen Linnea Pathway software (Life Technologies) (data not shown). Conversion of Illumina identifiers (Illumina_ID) to Uniprot IDs and removal of redundancies was performed by Da-Wei Huang, M.D., using DAVID Bioinformatics resource 6.7 software (<http://david.abcc.ncifcrf.gov>) (Da-Wei Huang, M.D., National Institute of Allergy and Infectious Diseases, personal communication) [35, 36]. The secretome data set containing up- or downregulated proteins (≥ 2 -fold), including proteins identified only in IFN- γ -, LPS-, or RTL1000-treated MAPC samples, was analyzed by Ingenuity Pathway Analysis (IPA) version 9.0 (Ingenuity Systems, Mountain View, CA, <http://www.ingenuity.com>). Statistically significant input protein identifications and IPA were mapped with a continuously updated and curated database of published literature regarding a range of functions, cellular locations, canonical pathways, and disease inter-relationships. The association between proteins in the data set and canonical pathways in the Ingenuity Pathways Knowledge Base was measured as a ratio of the number of molecules from the data that map to a pathway divided by the total number of molecules that map to the canonical pathway. A right-tailed Fisher's exact test was used to calculate the *p* value of the probability that the association between each protein in the data set and the canonical pathway was random [37].

RESULTS

MAPC Secretome Analysis

Human MAPCs were cultured for a minimum of three passages under defined conditions in the absence of any exogenous stimuli, and medium alone and MAPC-CM samples were collected at 24, 48, and 72 hours. A protein identified by LC-MS/MS analysis was deemed a confident match if at least two unique peptides were detected for that protein, and spectral counts obtained at all time points for each sample were averaged for downstream comparison between treatment groups. One of our long-term goals is to program coherent innate and adaptive immune responses *in vivo*. Toward this effort we determined the effect of potential licensing agents on the MAPC secretome. MAPC-CM was monitored over 72 hours of treatment with the three licensing agents IFN- γ (2 ng/ml), LPS (10 μ g/ml), and RTL1000 (5 μ g/ml) [17–20]. Proteins were considered to be MAPC secretome candidates if more than 2.5 unique peptides were identified in any of the treatment group samples, disallowing any proteins with more than 2.5 unique peptides identified in medium-alone

samples. The single exception to this cutoff criterion was thrombospondin-1. Although it was detected in medium (3.8 spectral counts), it was included in our analysis because it showed a 7.5-fold or greater increase in spectral counts in MAPC-CM. A wide range of functionally distinct classes of molecules was identified by high-performance liquid chromatography-MS/MS (Table 1), and the complete data set of 97 proteins identified is presented in supplemental online Table 1. Our data set includes 66 classically secreted proteins, 3 glycosphosphatidylinositol-linked proteins, 4 single-pass type I membrane proteins, and 24 proteins that are classically identified as endoplasmic reticulum or cytoplasmic residents but that have been identified from previous studies as being secreted via various nonclassic routes or by ectodomain shedding mechanisms.

MAPCs exposed to these agents showed a wide range of responses as reflected in the secretome (Fig. 1). Correlation curves plotting the averaged spectral counts of technical replicates at 24 hours ($n = 2$) suggested that all three treatments perturbed the MAPC secretome (Fig. 1C). When a twofold ratio change cutoff was applied to the 97 proteins in the MAPC secretome candidate list, IFN- γ , LPS, or RTL1000 treatment resulted in approximately 72% ($n = 69$) of the MAPC secretome candidates being of differential abundance. Of the 97 proteins detectable in the MAPC secretome at 24 hours, 24 were still detectable following IFN- γ treatment, 26 following LPS treatment, and 24 following RTL1000 treatment (Fig. 1A). Combined, 16 of the 97 proteins (17%) were detectable under all conditions. At 24 hours, IFN- γ treatment (2 ng/ml) [18] was associated with a decrease in abundance of 10 proteins and an increased abundance of 16 proteins. LPS (10 μ g/ml) [17] was associated with a decrease in abundance of 11 proteins and an increased abundance of 41 proteins. RTL1000, a CD74 ligand (5 μ g/ml) [20] was associated with a decrease in abundance of 10 proteins and an increased abundance of 26. The results indicate that MAPCs are able to recognize these stimuli and alter their protein secretion programs.

We used a combination of pathways analysis (Ingenuity IPA) and brute-force data mining coupled with structure-function homology modeling (Sybyl-X; Tripos, Inc., St. Louis, MO, <http://www.tripos.com>) to map interactions of the MAPC secretome proteins identified (Fig. 2). Forty-six of these proteins (47%) physically interacted with each other, clustering into two major groups (28 of 46, 61%; and 14 of 46, 30%). The proteins that directly interacted with each other are indicated by black lines in Figure 2, and the proteins that interacted as substrate/catalyst pairs are highlighted with substrates indicated by the arrowheads. Inclusion of indirect interactions (represented by dotted lines) expands the interactome to 58 of 97 (60%) of the MAPC secretome proteins. This array of interactions documents the complex interconnectedness and interdependence of the secretome. A three-dimensional workspace consisting of the ECM overlaid with the known interactions of MAPC secretome proteins identified is presented in Figure 3.

DISCUSSION

Our data document that MAPCs secrete molecules that regulate the biological activity of the ECM as well as secreting chemokines, cytokines, and an array of molecules participating in and regulating diverse programs, including angiogenesis/angiostasis,

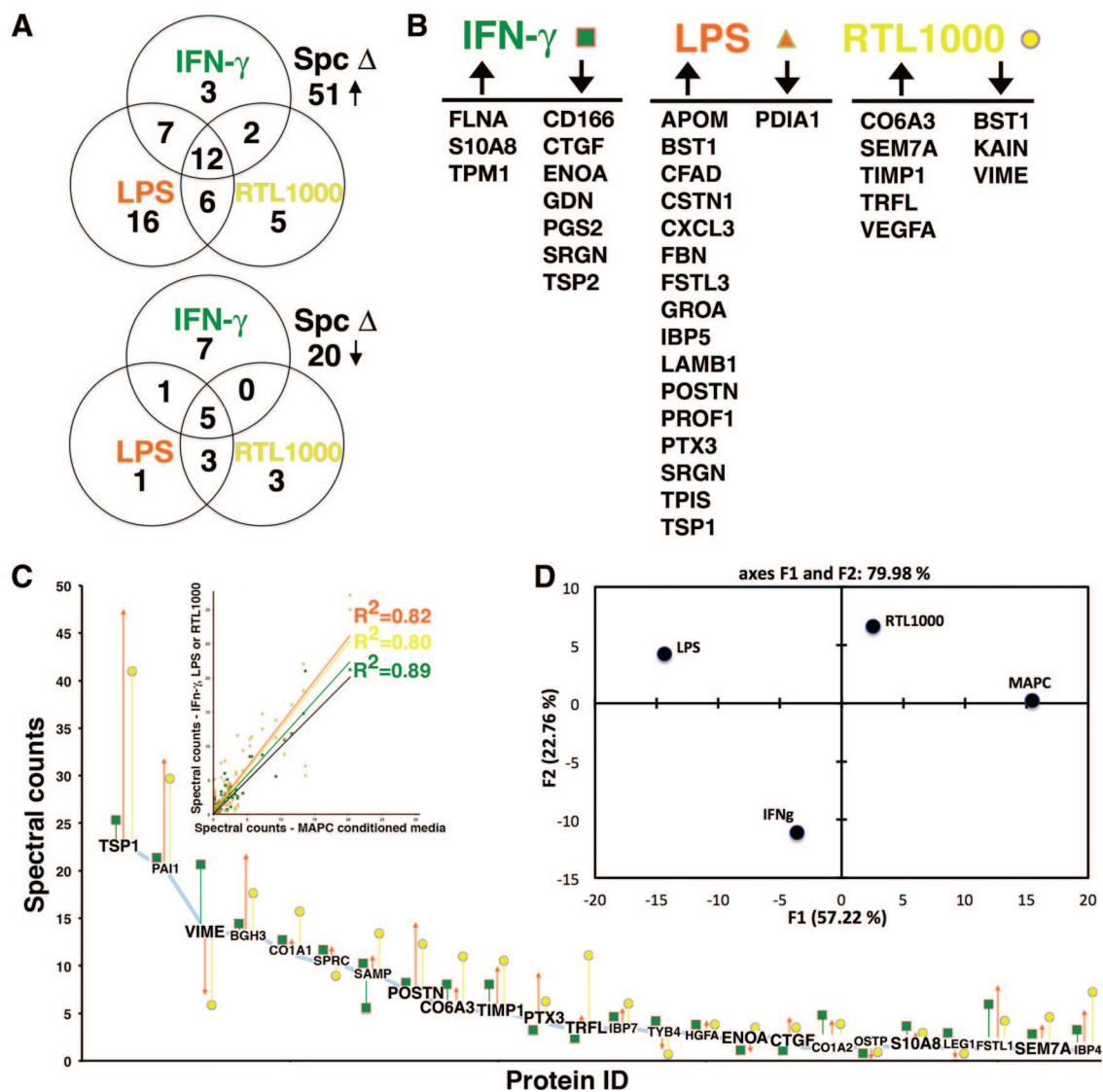


Figure 1. IFN- γ , LPS, and RTL1000 induced upregulation and downregulation of MAPC secretome proteins at 24 hours. **(A):** Venn diagram showing the overlap, relationship, and spectral count changes of 71 MAPC secretome candidates. **(B):** Treatment signatures as defined by unique Spc Δ . **(C):** Highest abundance secretome proteins and Spc Δ following treatment. The blue line indicates baseline abundance (MAPCs, no treatment). Larger font indicates uniquely up- or downregulated secretome proteins. Inset: Correlation curves plotting the averaged spectral counts of technical replicates at 24 hours ($n = 2$). **(D):** Principal component analysis of the 97 detected proteins. Abbreviations: IFN, interferon; LPS, lipopolysaccharide; MAPC, multipotent adult progenitor cell; Spc Δ , spectral count changes; VEGFA, vascular endothelial growth factor A.

targeted delivery of growth factors, and the matrix metalloprotease cascade (Table 1). Molecules that regulate the biological activity of the ECM include proteins that make up the ECM itself, such as type I and VI collagens, and proteins that regulate its construction/deconstruction. These include proteases such as MMP1, MMP2, and cathepsin B. Also detected were protease inhibitors and regulators of protease activation, including serglycin, kallistatin, TIMP1 and TIMP2, plasminogen activator inhibitor-1 (PAI-1), glia-derived nexin, PCOC1, and histidine-rich glycoprotein. A wide array of collagen-binding proteins are secreted by MAPCs, including biglycan, decorin, BIGH3, periostin, versican, and osteopontin. The MAPC secretome contains proteins that modulate the activity of growth factors and serve to attach and de-attach growth factors from ECM components for redistribution upon appropriate stimulation. These regulatory proteins include secreted protein acidic and rich in cysteine, latent

transforming growth factor binding proteins, follistatin-related proteins, vasorin, and thrombospondins. Additionally, MAPCs secrete innate immune system components that provide early protection from pathogens, such as pentraxins and lactotransferrin, and immunomodulatory proteins, including soluble intercellular adhesion molecule 1 (sICAM1), semaphorins, and galectins. It is important to note that many of the proteins identified in the MAPC secretome are multifunctional proteins that serve overlapping and alternative functions that depend upon variable signals from the local microenvironment.

The high abundance of protease inhibitor PAI-1 is consistent with identification of tryptic fragments from the intact activation peptide of plasminogen, suggesting that the plasminogen identified in the samples is (at least partially) in the zymogen (inactive) state (supplemental online Fig. 1). In addition, consistent with the high abundance of TIMP-1, a high-affinity regulator of

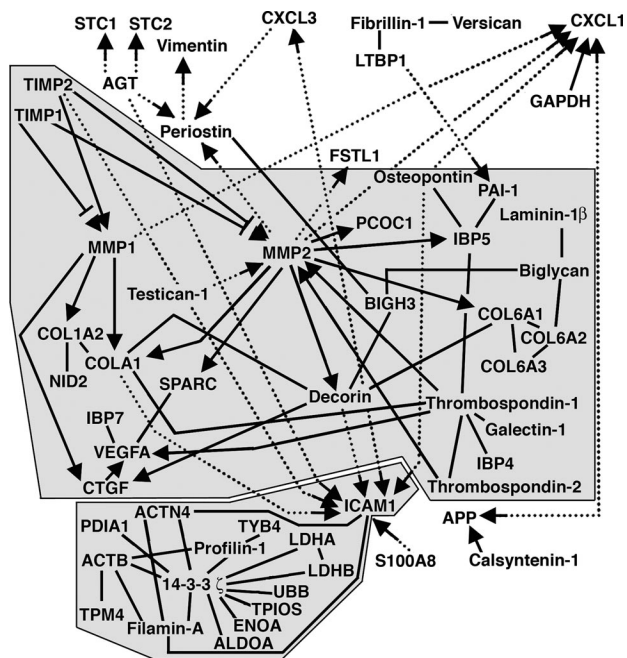


Figure 2. Known interactions between proteins identified in multipotent adult progenitor cell secretome. Proteins identified that directly interact with each other are indicated by solid black lines. Substrate/catalyst pairs are highlighted with substrates indicated by arrowheads. Indirect interactions are represented by dotted lines. Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LTBP, latent transforming growth factor binding protein; MMP, matrix metalloproteinase; PAI, plasminogen activator inhibitor; SPARC, secreted protein acidic and rich in cysteine; VEGFA, vascular endothelial growth factor A.

MMP2 activity, the MMP2 identified in the MAPC secretome is also (at least partially) in its zymogen form. This is inferred from the identification of tryptic peptide fragments that traverse the propeptide-mature protein boundary, with propeptide tryptic peptide fragments representing 78% coverage of the propeptide domain versus 37% coverage of the mature MMP2 protein (supplemental online Fig. 2). The data are consistent with the concept that MAPCs secrete an array of factors that maintain the ECM in a state of repose, ready to respond appropriately to exogenous stimuli that would be expected to be generated as a result of local physical injury of tissue or infiltration by various cell types.

IFN- γ is a cytokine secreted by natural killer cells, monocytes/macrophages, and Th1 CD4⁺ cells, and it would be expected to be present in a local environment as a result of infiltrating leukocytes. LPS could be present in a local environment as a consequence of a bacterial infection. Cellular trafficking into inflammatory compartments results in proteolytic ectodomain-shedding of membrane proteins, including fragments of MHC class I and MHC class II molecules. We hypothesize that these fragments regulate local inflammatory processes. In our recently published studies, we documented that RTL1000, a recombinant molecule consisting of the β -1 and α -1 domains of MHC class II, binds and downregulates CD74, inhibiting leukocyte trafficking and inflammation, at least in part via decreased macrophage inhibitory factor signaling [20]. Consequently, RTL1000 was tested for the first time in experiments presented in this study to determine whether MAPCs could respond to this mimic of a highly localized tolerogenic mechanism. Our data documented

that MAPCs were able to recognize these three stimuli with concomitant differential alteration of their protein secretion programs.

One of the major challenges in fully characterizing a secretome is that many of the molecules secreted and expected to be active in situ in cell-to-cell signaling interactions function at sub-nanogram to picogram levels, and these are difficult to detect using LC-MS/MS against a background of thousands of highly abundant serum proteins (in the mg/ml range) [38]. We used an immunodepletion strategy to remove the top 14 background serum components [39]. Having detected galectin-1, CXCL1, and CXCL3 in the MAPC secretome (Table 1), the level of detection we reached with our approach is estimated to be proteins present at concentrations of approximately 200 pg/ml [40]. In future studies we will incorporate selective enrichment technology that will allow detection of more of the lower abundance proteins expected to be present in the MAPC secretome [41]. A second caveat when analyzing this data set is that a protein was deemed a secretome candidate if more than 2.5 unique peptides were identified in any of the biological replicates under any of the conditions used, and thus this study was not designed to address the variation between biological replicates. The data accurately reflect the differential effects of IFN- γ , LPS, and RTL1000 on MAPCs cultured from a single individual donor.

Bioinformatics and pathways analysis were used to evaluate in detail how a representative subset of molecules present in the MAPC secretome could potentially function as a complex network of interacting molecules that regulate the ECM when MAPCs differentially respond to IFN- γ , LPS, and RTL1000. From a wound-healing perspective, the differential responses observed indicate that MAPCs could respond rapidly and efficiently to various types of insults and alterations that occur as a function of time that are induced in part by infiltrating inflammatory and immune cells. Molecules identified in the MAPC secretome have the ability to modulate the behavior of infiltrating cells, and pathways analysis suggests that the molecular components are integrated on a number of levels, with MAPCs secreting a complex mixture of collagens and collagen-interacting molecules. We hypothesize that this network of interacting molecules secreted by MAPCs serves to guide a simultaneously coordinated plethora of events. The events potentially include recruitment of cells (via CXCL1 and CXCL3, for example) and managing the behavior of infiltrating cells by regulating their effector functions (secretion of galectin-1 negatively regulating T-cell effector function, for example) appropriately for a wound-healing environment [42]. In addition, our data document that the MAPC secretome contains molecules that prevent opportunistic pathogens from spreading and enhancing tissue damage. MAPCs secrete molecules that regulate access of tissues by macrophages for clearing of debris while simultaneously coordinating with top-level memory/effector CD4⁺ and CD8⁺ cells, using their cytokine and chemokine arsenal to communicate and program the systemic response to specific stimuli.

Cellular therapeutics are rapidly entering the clinical arena. Establishing dose-response, defining optimal routes of delivery, and establishing potency are essential to the optimization of clinical application. All the steps first await a thorough understanding of the primary mechanisms by which MAPCs provide clinical benefit.

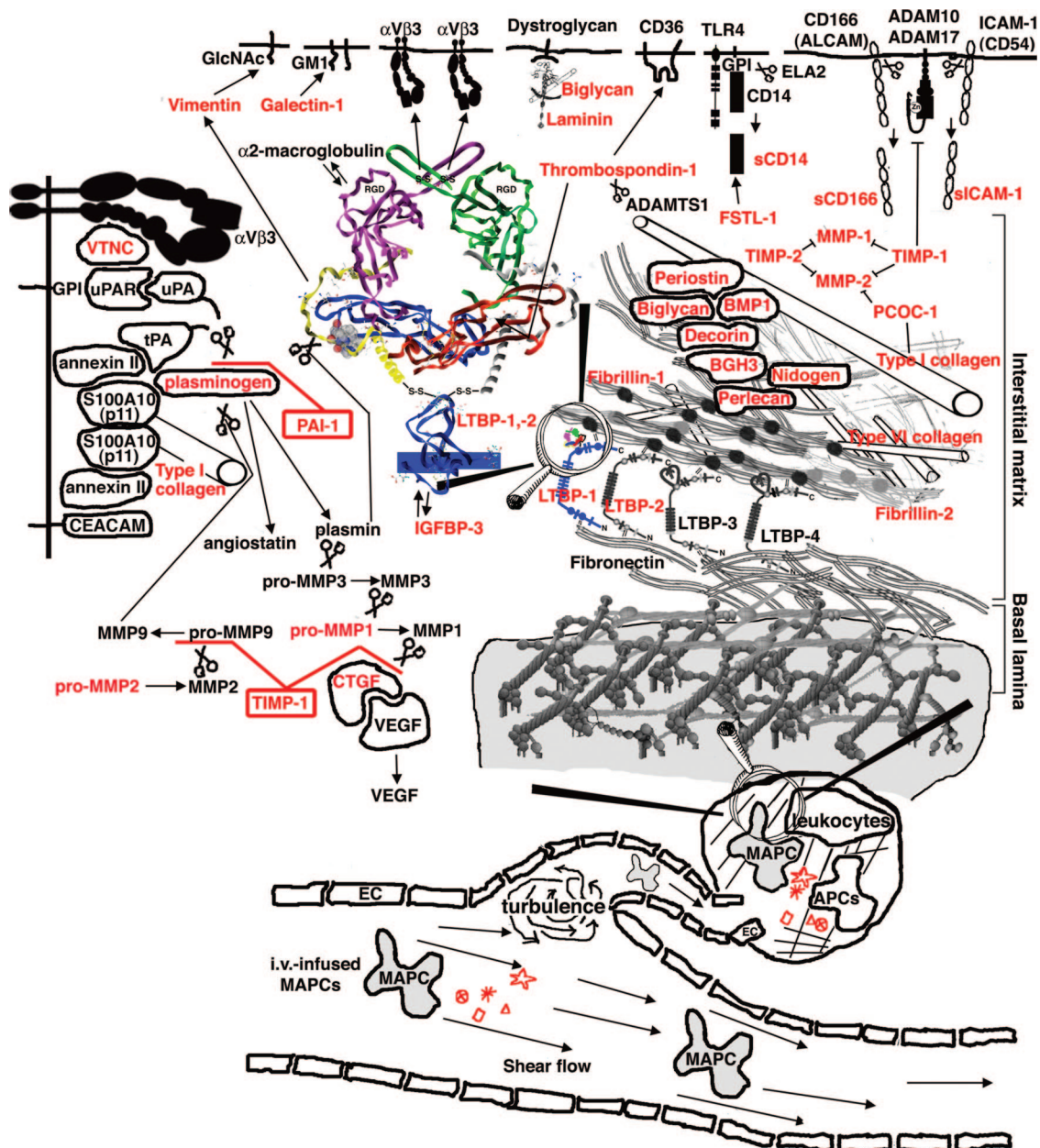


Figure 3. A schematic overview of our hypothesis. Intravenously delivered MAPCs and secretome components identified (red) are hypothesized to regulate broad aspects of growth factor delivery, the metalloproteinase cascade, and angiogenesis. Abbreviations: LTBP, latent transforming growth factor binding protein; MAPC, multipotent adult progenitor cell; MMP, matrix metalloproteinase; PAI, plasminogen activator inhibitor; uPA, urokinase plasminogen activator; uPAR, urokinase plasminogen activator receptor; VEGF, vascular endothelial growth factor.

In our previously published studies it was documented that rodent and human MAPCs are safe and can modulate the immune system via the secretion of soluble factors [15, 16]. Good manufacturing practice-manufactured human MAPCs are now in phase II clinical trials for inflammatory bowel disease, acute myocardial infarction, and ischemic stroke. Two phase I clinical trials (safety studies) have been completed using MAPCs for prevention of GVHD and for treatment of acute myocardial infarction [43, 44]. The beneficial effects of MAPCs *in vivo* may be the product of multiple mechanisms, including a proangiogenic effect through trophic support [45–47] and the modulation of the immune response [15, 48]. Immunoregulatory properties of MAPCs

may be important for GVHD prevention and treatment. Human and rat MAPCs inhibit allogeneic T-cell proliferation mediated by soluble factors that include indoleamine 2,3-dioxygenase [16]. At present, it is still unclear how many different molecular pathways are impacted. This study was designed to begin to catalog the array of primary targets.

The route of delivery also remains unclear and may be critical for optimal application of stromal cell therapies. Our focus has been on exploiting the immunomodulatory potential of MAPCs to protect patients from GVHD following allogeneic hematopoietic stem cell transplantation [16, 49]. Most studies have treated patients with systemic intravenous administration in which

stromal cells first passage to the lung where they are initially retained [48, 50]. In one study, lung entrapment appeared to be required for treatment of peritoneal adhesion [51]. In other models, for example, chemical injury to the corneal epithelial surface, either local or systemic delivery of MSCs ameliorates damage [52]. These reports reflect the ongoing uncertainty regarding how clinical benefit is achieved. Finally, recent studies evaluating encapsulated stromal cell product support the concept that stromal cell homing to specific organs is not absolutely required to control inflammation [53]. These observations support the concept that MAPC immune regulation is a consequence of soluble factors that act systemically.

CONCLUSION

Our studies used a proteomics approach to characterize and quantify the changes induced in the MAPC secretome over 72 hours in vitro under steady-state conditions and in the presence of the inflammatory triggers IFN- γ and LPS, or a tolerogenic CD74 ligand, RTL1000. MAPCs differentially responded to each of the tested stimuli, and these studies provide a foundation for understanding and controlling the function of MAPCs. As such, the data presented here provide a strong background for hypothesis-driven experiments assessing MAPC response to molecular and cellular triggers and events for eventual programming of MAPCs to be used in a wide range of clinical applications.

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AUTHOR CONTRIBUTIONS

G.G.B.: conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing, final ap-

proval of manuscript; W.v.H.: conception and design, data interpretation, manuscript writing; L.F.N.: conception and design, manuscript writing; A. Reddy and L.L.D.: collection of data, data analysis and interpretation, manuscript writing; P.A.W.: assembly of data, data analysis, manuscript writing; A. Raber: collection of data, manuscript writing; A.B. and J.P.: data analysis and interpretation, manuscript writing; R.J.D.: data interpretation, manuscript writing; R.T.M.: conception and design, financial support, data analysis and interpretation, manuscript writing, final approval of manuscript.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

G.G.B. is the inventor of the RTL technology, has uncompensated intellectual property rights with Artielle ImmunoTherapeutics, Inc., and is a compensated consultant with Virogenomics, Inc. G.G.B. and Oregon Health and Science University (OHSU) have a significant financial interest in Artielle ImmunoTherapeutics, Inc., a company that may have a commercial interest in the results of this research and technology. This potential conflict of interest has been reviewed and managed by the OHSU Conflict of Interest in Research Committees. W.v.H. is a compensated Athersys employee, has compensated intellectual property rights, and is a compensated stock holder. A. Raber is a compensated Athersys employee and has compensated stock options from Athersys. A.B. is a compensated Athersys employee. J.P. is a compensated Athersys employee, is head of research and development at Athersys, and has compensated stock options in Athersys. R.J.D. is a compensated Athersys employee, is an uncompensated patent holder with Athersys, and has compensated stock options in Athersys. Athersys has licensed OHSU's rights in intellectual property coinvented by R.T.M., which may apply to the use of MAPCs in this treatment area. This potential individual and institutional conflict of interest has been reviewed and managed by OHSU. W.v.H. and R.J.D. are coinventors of the MultiStem technology.

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