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"*Generation of Functional Macrophages from Adult Mesenchymal Stem Cells*"

D i s s e r t a t i o n

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vorgelegt von Eva Freisinger
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Dokumentationsblatt

Die vorliegende Dissertation ist eine der Grundlagenforschung zuzuordnende experimentelle Arbeit. Die Autorin beschäftigt sich mit dem Differenzierungspotential adulter mesenchymaler Stammzellen. Beginnend mit einem einleitenden Kapitel zur Vermittlung von Basiswissen und Hintergrundinformationen soll der Leser in das Forschungsgebiet der adulten Stammzellforschung eingeführt werden. Es folgt eine detaillierte Beschreibung des Projektes: Mittels eines im Rahmen der vorliegenden Arbeit entwickelten Differenzierungsmediums wird erstmals die Generierung hämatopoetischer Zellen aus mesenchymalen Stammzellen des Fettgewebes *in vitro* beschrieben. Es schließt sich eine umfassende Charakterisierung dieser neuartigen Zellart mit Hilfe genetischer, immunologischer und funktioneller Analysen an. Unter Darlegung der Ergebnisse in zahlreichen Abbildungen und graphisch-schematischen Darstellungen wird eine Ähnlichkeit der differenzierten Stammzellen mit Makrophagen/ Monozyten nachgewiesen, was deren Zuordnung zur myeloischen Reihe erlaubt. Die Bedeutung dieser Entdeckung und eine Vorstellung potenzieller zukünftiger Anwendungsgebiete im Rahmen von Forschung und angewandter Zelltherapie werden abschließend zur Diskussion gestellt. Insbesondere wird hierbei ein Einblick in erste eigene, sich dem vorgestellten Projekt anschließende Forschungsergebnisse gewährt.

Die vorliegende Arbeit wurde veröffentlicht

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Zusammenfassung

Mesenchymale und hämatopoetische Gewebe sind wichtige Reservoirs adulter Stammzellen. Das Potenzial gewebeständiger mesenchymaler Stammzellen (MSCs) in Zellen der mesodermalen und ektodermalen Linie zu differenzieren, ist bekannt. Die Hypothese, dass adhärente adipogene mesenchymale Stammzellen (ASCs) zu Zellen mit hämatopoetischen Charakeristika differenziert werden können, wurde im Rahmen dieser Arbeit untersucht. Unter Kulturbedingungen in eigens dafür entwickeltem Differenzierungsmedium, welches die Interleukine IL-1β, IL-3 und den Monozytenkolonien-stimulierenden Faktor M-CSF als wirksame Stimuli beinhaltet, entwickeln klonale ASCs hämatopoetische funktionelle und strukturelle Eigenschaften. Die hämatopoetisch differenzierten Zellen (HD) exprimieren frühe hämatopoetische (c-kit, PROM1, CD4) als auch Monozyten/ Makrophagen-spezifische Marker (CCR5, CD68, MRC1, CD11b, CSF1R). Immunzytochemie belegt das Vorkommen von CD68, NOS2 und CSF1R auf Proteinebene. Zudem lassen sich in HD-Zellen funktionelle Eigenschaften von Makrophagen, wie die Fähigkeit zur Phagozytose (zu >95%) oder enzymatische Aktivität von α-Naphthyl-Acetat-Esterase (zu 36 %) nachweisen. HD-Zellen zeigen eine Reaktion auf Stimulation mit LPS und IL-4 als Aktivatoren des klassischen bzw. des alternativen Reaktionsweges der Immunantwort, indem sie verstärkt CD14 und HLA-DRB1 exprimieren und IL-2, IL-10 und TNF sezernieren.
Zusammenfassend betrachtet stellen die hier vorgelegten Ergebnisse eine neuartige Generierung funktionsfähiger Makrophagen aus adipogenen mesenchymalen Stammzellen *in vitro* vor. Daraus könnten sich neue Möglichkeiten für einen zukünftigen klinischen Nutzen in der Zelltherapie mit adipogenen Stammzellen ergeben.

Schlüsselwörter: Mesenchymale Stammzelle, Hämatopoetische Dfferenzierung, Makrophage, Monozyt, Phagozytose

Summary

Mesenchymal and hematopoietic tissues are important reservoirs of adult stem cells. The potential of tissue resident mesenchymal stem cells (MSCs) to differentiate into cells of mesodermal and ectodermal lineages has been reported previously. In the following, the hypothesis that adherent adipose tissue resident mesenchymal stem cells (ASCs) are capable of generating cells with hematopoietic characteristics has been examined. When cultured in a specifically developed differentiation media containing IL-1β, IL-3 and M-CSF, clonally isolated ASCs develop into cells with hematopoietic attributes. The hematopoietic differentiated cells (HD) express early hematopoietic (c-kit, PROM1, CD4) as well as monocyte/ macrophage markers (CCR5, CD68, MRC1, CD11b, CSF1R). Immunocytochemistry verified CD68, NOS2 and CSF1R on the proteomic level. Additionally, HD cells display functional characteristics of monocyte/ macrophages such as phagocytosis (>95%) and enzymatic activity of α-Naphthyl Acetate Esterase (36.26%). HD cells are also responsive to stimulation by IL-4 and LPS as activators of both, classical and alternative pathway of the immune response, shown by increased CD14 and HLA-DRB1 expressions and release of IL-2, IL10, and TNF.

In summary, this study characterizes the novel potential of adipogenic mesenchymal stem cells to generate functional macrophages *in vitro*, and therefore could pave a way for their potential use in cell therapy applications.

Key Words: Tissue resident Mesenchymal Stem Cell, Hematopoietic Differentiation, Macrophage, Monocyte, Phagocytosis

Abbreviations

%	Percent
°	Number
°C	Celsius degree
1-MTG	1-Mono-thioglycerol
A	
A	Adenosine
Ab	Antibody
AIDS	Aquired Immune Deficiancy Syndrome
ANOVA	Analysis of Variance
APC	Antigene presenting cell
ASCs	Adipose-tissue derived MSCs
B	
B-cells	B-Lymphocytes
BHA	Butylated hydroxyanisole
BMCs	Bone marrow cells
bp	Base pair
C	
C	Cytosine
CCD	Charge-coupled device
CD	Cluster of Differentiation
cDNA	Complementary DNA
CT	Threshold Cycle
D	
DMEM	Dulbecco's Modification of Eagle's Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
E	
E. coli	Escherichia Coli
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ESCs	Embryonic stem cells
et al.	Et alii; and others
EtOH	Ethanol
F	
FBS	Fetal Bovine Serum
Fc	Fragment crystallizable region
Fig.	Figure
G	
g	Gramm
G	Guanine
G1/G2	Interphase 1/2 ratio
gag	Group specific antigen
GAPDH	Glyceraldehyde 3-phosphate
H	
h	human
HD	Hematopoietic differentiated ASCs
HE	Hematoxylin-Eosin
HeNe	Helium Neon Laser
HGF	Hepatocyte growth factor
HI	Heat inactivated
HIV	Human Immunodeficiancy Virus
HIV-HD	HIV-infected HD
HLA	Human leukocyte antigen
HSCs	Hematopoietic Stem Cells
I	
Ig	Immune globulin
IL	Interleukine
IRB	Institutional Review Board
L	
LPS	Lipopolysaccharide
M	
m	Metre
m	milli; 10^{-3}
µ	micro; 10^{-6}
M	Molar
M-CSF	Macrophage colony-stimulating factor
MAP	Microtubule-associated protein
MEM	Minimum Essential Media
mRNA	Messenger RNA
MSCs	Mesenchymal Stem Cells
N	
n	nano; 10^{-9}
NFkB	Nuclear factor kappa B
NK-cells	Natural killer cells
NO	Nitric oxide
no.	Number
NOS	Nitric oxide synthetase
NSE	Naphthyl acetate esterase
O	
O.D.	Optical Density
OSM	Oncostatin M

P	
p	pico; 10^{-12}
p24	Protein 24
p-value	Statistical significance
PBS	Phosphate- Buffered Saline
PI	Propidium Iodide
R	
rh	Recombinant human
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT	Room Temperature
rt RT-PCR	Real-time RT-PCR
RT-PCR	Reverse Transcription PCR
S	
S-phase	Synthesis phase
SD	Standard derivation
STD	Sexually transmitted disease
SVF	Stromal vascular fraction
T	
T	Temperature
TAE	Tris-Acetate-EDTA buffer
T-cell	T-Lymphocyte
Tm	Melting Temperature
TNF	Tumor necrosis factor
TU	Transducing Unit
TX-100	Triton-X 100
U	
U	Unit

Einleitung

In der modernen Medizin stellt die Verwendung von Stammzellen eine innovative Methodik verbunden mit weitreichenden Möglichkeiten zur Regeneration spezifischer körpereigener Zellen und Gewebe dar. Diese ist in dem hohen Differenzierungspotential der noch nicht determinierten Stammzellen und fehlender Immunogenität hinsichtlich einer autologen Transplantation begründet. Insbesondere embryonale Stammzellen zeigen ein hohes Maß an Wandelbarkeit mit der Fähigkeit zur Differenzierung in Zellen aller drei Keimblätter (Pluripotenz). Jedoch ist die Verwendung menschlicher embryonaler Stammzellen für Forschung und Medizin technisch aufwändig und ethisch umstritten („SKIP"-Argumentation). Umso mehr richtet sich der Fokus auf die primär multipotenten adulten Stammzellen, welche eine Differenzierung zu verschiedenen Gewebstypen innerhalb eines Keimblattes ermöglichen. Durch neue Methoden der De- und Trans- Differenzierung gibt es jedoch erste Ansätze, diese natürlichen Grenzen zu überschreiten und das Spektrum der Zielgewebe maßgeblich zu erweitern. Eine der vielversprechendsten adulten Stammzellpopulationen sind die Mesenchymalen Stammzellen (MSCs).

Mesenchymale Stammzellen

Mesenchymale Stammzellen sind gewebsständige adulte Progenitorzellen die dem mesodermalen embryonalen Keimblatt abstammen. Sie besitzen hohes Regenerationspotential bei multipotenter Differenzierungsfähigkeit.
Um eine einheitliche Nomenklatur festzulegen, schlug die Internationale Gesellschaft für Zelltherapie (ISCT) den Terminus „multipotente mesenchymale Stromazelle" für alle plastik-adhärent wachsenden Zellen unabhängig vom Isolationsgewebe vor. Der Terminus „Mesenchymale Stammzelle" ist belegt für Zellen, die zudem die folgenden Kriterien erfüllen [1,2] :

(I) Wachstumsadhärenz an Plastik
(II) Expression von CD73, CD90 und CD105 bei Abwesenheit von CD11b, CD14, CD 19, CD79*α*, CD45 und HLA-DR Expression
(III) Differenzierungspotential zu Adipozyten, Chondrozyten und Osteoblasten.

MSCs können aus verschiedenen Geweben isoliert werden, insbesondere aus Knochenmark (BMSCs), Fettgewebe (ASCs) und Muskulatur [3-5]. Neuere Studien

weisen darauf hin, dass sich Mesenchymale Stammzellen aus heterogenen Subpopulationen zusammensetzen, die sich hinsichtlich ihrer biologischen Eigenschaften unterscheiden. Ein direkter Vergleich zwischen humanen ASCs und BMSCs zeigte eine ca. 90% ige Übereinstimmung des Immunophänotyps [6]. Eingehendere Untersuchungen der Stammzellen dieser beiden verschiedenen Isolationsgewebe mittels Affymetrix Gen Chip fanden ein gemeinsames Transkriptom als Erklärung für die Ähnlichkeit (R. Izadpanah, B. Bunnell, C. Kriedt, unveröffentlichte Beobachtung, 2006) [7]. Der Korrelationskoeffizient zwischen den Transkriptomen von ASCs und BMSCs verschiedener Donoren liegt demnach bei etwa 50%, dies entspricht einem durchschnittlichen Korrelationskoeffizient von 71% (ASCs) bzw. 64% (MBSCs) indivdueller Donoren [8]. Dennoch gibt es Unterschiede in der Proteinexpression der Oberflächenantigene. So ist der Stammzellmarker CD34 bisher nur auf ASCs in frühen Passagen nachgewiesen worden, nicht jedoch auf BMSCs [9,10]. Neben CD34 gelten insbesondere CD44, CD73, CD90, CD105 als Standardmarker der ASCs. Trotz bestehender Abhängigkeit der Eigenschaften adipogener Stammzellen von Isolations- und Kultivierungsbedingungen [11,12]
ist der Immunphänotyp als relativ konstant beschrieben worden [13,14]

Ein spezifischer und eindeutiger genetischer Marker konnte bisher jedoch noch nicht identifiziert werden. Daher wird der Terminus „adipose-derived stromal/ stem cells“ nach einem Konsens der International Fat Applied Technology Society (IFATS) an Hand der Kriterien plastik-adhärentes Wachstum, Kultivierung und serielle Passage sowie Multipotenz von aus Fettgewebe isolierten Zellen verwendet .

Neben der Differenzierung zu Adipozyten, Osteozyten und Chondrozyten sind mit Hepatozyten und Myozyten weitere Zellen der mesodermalen Linie aus ASCs differenzierbar [15-18]. Mit der Entdeckung des neurogenen Differenzierungspotentials von adipogenen und knochenmarksständigen MSCs wurde darüber hinaus eine Differenzierung hin zu nicht-mesodermalen Linien belegt [19]. Diese Linien überschreitende Differenzierung als Zeichen höhergradiger Potenz der Stammzelle wird Transdifferenzierung genannt.
Eine Transdifferenzierung adipogener MSCs zu Zellen der hämatopoetischen Linie wurde bisher noch nicht belegt.

Hämatopoetische Differenzierung

Hämatopoetische Stammzellen (HSCs) als weitere Gattung adulter Stammzellen stammen ebenfalls von dem embryonalen Mesoderm ab und differenzieren zu diversen Blutzellen der myeloiden (Monozyten, Makrophagen, Neutrophile, Erythrozyten, Dendritische Zellen etc.) und lymphoiden Linie (T-Lymphozyten, B-Lymphozyten, NK-Zellen) [16]. Die Fähigkeit zur Hämatopoese besitzen neben HSCs neueren Studien zu Folge auch embryonale Stammzellen (ESCs) *in vitro* [20,21]. Es konnte gezeigt werden, dass aus ESCs differenzierte Makrophagen spezifische Oberflächenantigene, z.B. CD14, CD4, CXCR4 und HLA-DR, ausbilden [22]. Weiterhin tragen Adipozyten und undifferenzierte adipogene MSCs innerhalb des intakten Fettgewebes zum Anstieg proinflammatorischer Zytokin- Level [23] und vermutlich zur Generierung hämatopoetischer Zellen bei [24,25]. Zellen der Stroma-Gefäß-Fraktion (SVF), isoliert aus murinem Fettgewebe, rekonstituieren zudem effizient hämatopoetische Hauptlinien [25]. Die SVF besteht aus verschiedenen Zellen mit teils mesenchymalen (CD34+, CD90+, CD29+, CD44+, CD105+, CD117+), teils endothelialen Progenitorzell-Markern (CD34+, CD90+, CD44+, CD54+). Es wurde über hämangioblastäre Aktivität der SVF aus humanem Fettgewebe berichtet. Diese hämatopoetische Aktivität war allerdings auf die nicht-adhärente CD45-/KDR+/CD105+ Subpopulation der SVF begrenzt [26]. Ob auch adipogene mesenchymale Stammzellen hämatopoetisches Differenzierungspotential besitzen, soll im Folgenden untersucht werden.

Ziel der Studie

Im Rahmen der vorliegenden Arbeit sollte untersucht werden, ob adhärente MSCs, isoliert aus menschlichem Fettgewebe, Transdifferenzierungspotential hin zur hämatopoetischen Linie besitzen (HD Zellen). Es soll insbesondere geprüft werden, ob unter speziellen Kulturbedingungen Zellen generiert werden können, die spezifische strukturelle und funktionelle Eigenschaften von Monozyten/ Makrophagen aufweisen. Es würde sich somit eine effiziente Methode zur Gewinnung von Zellen hämatopoetischer Beschaffenheit aus multipotenten mesenchymalen Stammzellen durch klonale Expansion entwickeln lassen. Auf Basis dieser Entdeckung könnte Fettgewebe als reichlich verfügbare und leicht zugängliche Ressource für adulte

Stammzellen potenziell der Regeneration hämatopoetischer Zellen dienen. Dies könnte zukünftig im Rahmen der Zelltherapie insbesondere zytopenischer hämatologischer und immunologischer Erkrankungen Bedeutung erlangen.

Introduction

In the domain of modern medicine, the usage of stem cells poses as an innovative method combined with extensive options to regenerate specific cells and tissues of the human body. This is due to the high differentiation potential of the still undetermined stem cells and its lack of immunogenicity with regard to autologous grafting. Especially embryonic stem cells show a high degree of alterability combined with their competence to differentiate into cells of all three germ layers (pluripotency). However, the use of human embryonic stem cells in research and applied medicine is technically complex and ethically contentious. All the more, the primarily multipotent adult stem cells get focused, that allow a differentiation towards various tissues within the same germ layer. Via new methods of de- and trans-differentiation, there are first approaches available to exceed these natural limits and to extend the range of destined tissues. Regarding that, mesenchymal stem cells represent one of the most promising adult stem cell populations.

Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs) have been described as tissue resident adult progenitor cells that develop from the embryonic mesodermal germ layer. MSCs have a high capacity of self-renewal while maintaining their multipotent differentiation potential.

To define a consistent nomenclature, the International Society for Cell Therapy (ISCT) proposed the term “multipotent mesenchymal stromal cell” for all plastic-adherent growing cells regardless their origin tissue. The term “mesenchymal stem cell” is assigned for cells that additionally fulfill the following criteria [1,2] :

(1) plastic adherence

(2) expression of CD73, CD90 and CD105 but lack of CD11b, CD14, CD19, CD79*α*, CD45 and HLA-DR expression

(3) differentiation potential towards adipocytes, chondrocytes, osteoblasts.

MSCs have been isolated and characterized from a variety of tissues including bone marrow, adipose tissue, and muscle [3-5]. Recent studies indicate that mesenchymal stem cells consist of heterogeneous subpopulations which differ concerning their biological properties. A direct comparison between human ASCs and BMSCs

showed a ca. 90% conformity of the immune-phenotype [6]. Detailed analysis of the stem cells isolated from those two different tissues by using affymetrix gen chips revealed a common transcriptome that explains the similarities (R. Izadpanah, B. Bunnell, C. Kriedt, unpublished observation, 2006) [7]. The correlation coefficient of the transcriptomes of ASCs and BMSCs ranges at about 50% for multiple donors which compares to an average correlation coefficient of 71% (ASCs) and 64% (BMSCs) between individual donors [8]. However, there are differences in protein expression of the surface antigens, e.g. the stem cell marker CD34 has been detected on ASCs in early passages but so far it has not on BMSCs [9,10]. Beside CD34, especially CD44, CD73, CD90 and CD105 are classified as standard genetic markers of ASCs. Despite the dependence of the characteristics of adipogenic stem cells on isolation and cultivation conditions [11,12], the immune-phenotype is reported to remain relatively constant [13,14].

So far, a specific and biunique genetic marker could not be identified. Thus, the International Fat Applied Technology Society (IFATS) reached a consensus to adopt the term “adipose-derived stromal/stem cells” for the plastic-adherent, cultured and serially passaged, and multipotent cell population from adipose tissue.

Aside from the differentiation into adipocytes, osteocytes and chondrocytes, hepatocytes and myocytes as further cells of the mesodermal lineage can be developed from ASCs [15-18]. By discovering the neurogenic differentiation potential, it has been confirmed that MSCs derived from bone marrow (BMSCs) and adipose tissue (ASCs) can evolve into non-mesodermal lineages as well [19]. The lineage crossing differentiation as a sign for higher potency of the stem cell is called trans-differentiation. A trans-differentiation of adipogenic MSCs towards cells of the hematopoietic lineage has not been documented yet.

Hematopoietic differentiation

Hematopoietic stem cells (HSCs) as a further type of adult stem cells do also originate from the embryonic mesodermal germ layer and they are committed to form blood cell types including myeloid (monocytes and macrophages, neutrophils, erythrocytes, dendritic cells, etc.) and lymphoid lineages (T-cells, B-cells, NK-cells)

[16]. In addition to HSCs, several studies have demonstrated the hematopoietic differentiation potential of embryonic stem cells (ESCs) *in vitro* [20,21]. It has been shown that ESC derived macrophages displayed specific cell surface markers such as CD14, CD4, CCR5, CXCR4, and HLA-DR [22]. It has also been reported that adipocytes and/ or undifferentiated ASCs within the intact adipose tissue contribute to an elevation of proinflammatory cytokine level [23] and may support hematopoietic cell generation [24,25]. In addition, transplanted cells from the stromal-vascular fraction (SVF) isolated from murine adipose tissue has been shown to efficiently reconstitute major hematopoietic lineages [25]. The SVF contains different cell populations expressing MSC markers including CD34, CD90, CD29, CD44, CD105, and CD117 and endothelial-progenitor-cell markers including CD34, CD90, CD44, and CD54. The SVF of human adipose tissue has been identified to exhibit hemangioblastic activity. However, this hematopoietic performance was restricted to the CD45-/KDR+/CD105+ cells among the non-adherent subset in the SVF [26]. If also adipogenic mesenchymal stem cells exhibit hematopoietic differentiation potential will be investigated in the following.

Aim of the Study

In this study, it should be examined if adherent MSCs derived from human adipose tissue are able to trans- differentiate into cells of the hematopoietic lineage (HD cells). It should in particular be verified whether it is possible to generate cells under specific culturing conditions that exhibit distinct characteristics and functionality of monocytes/ macrophages. If possible, an efficient method to generate cells with hematopoietic nature from ASCs through expansion of clonally isolated multipotent mesenchymal cells would be developed. This novel finding could potentially lead to define new conditions for amplifying hematopoietic cells from adipose tissue as an abundant and readily accessible source of stem cells. Regarding cytopenic hematological and immunological disorders, this could gain importance as a possible future tool for cell therapies.

Materials

(a) Cells and Media

human ASCs

The human ASCs were isolated from subcutaneous adipose tissue of healthy donors* based on standard protocols; fat tissue was provided by Dept. of Surgery, Tulane University Health Science Center, NO, LA. The collection of all human tissue samples was approved by Institutional Review Board (IRB) of Tulane University, School of Medicine, New Orleans, Louisiana.

human BMCs

The human BMSCs were obtained from bone marrow samples of healthy donors*; Cells were provided by Dept. of Pharmacology, Tulane University Health Science Center, NO, LA

*healthy donor = normal BMI, no diabetes, no cancer in history, non infectious, age 18 – 35 years old

(a) Media

ASC Maintenance Medium		**ASC Freezing Medium**	
α -MEM		α -MEM	45%
FBS (HI)	20%	FBS (HI)	50%
L-Glutamine	1%	DMSO	5%
Penicilline/ Streptomycin 1:1	1%	L-Glutamine	1%

Adipogenic Differentiation Medium	
α-MEM	
Dexamethasone	0.5μM
Isobutylmethylxanthine	0.5mM
Indomethacin	50μM
FBS (HI)	20%
Penicilline/ Streptomycin 1:1	1%
L-Glutamine	1%

Chondrogenic Differentiation Medium

StemPro Chondrogenesis Differentiation Kit

Hematopoietic Differentiation Medium	
α-MEM	
FBS (HI)	10%
1-Monothioglycerol	0.1µl/ml
IL-1ß	100U/ml
IL-3	500U/ml
M-CSF	20U/ml

Hepatogenic Differentiation Medium	
α-MEM	
Dimethyl sulfoxide	0.1%
rhHGF	10ng/ml
rhOSM	10ng/ml

Neurogenic Differentiation Medium	
DMEM with BHA	200µM final concentration
KCl	5mM
Valproic acid	2µM
Forskolin	10µM
Hydrocortisone	1µM
Insulin	5g/ml

Osteogenic Differentiation Medium	
α-MEM	
Dexamethasone	1nM
Ascorbate-2-phosphate	50µM
β-glycerophosphate	2mM
FBS (HI)	20%
Penicilline/ Streptomycin 1:1	1%
L-Glutamine	1%

Tab. 1

(b) Staining solutions

Differentiation	Staining
Adipogenesis	Oil Red O solution
Chondrogenesis	Toluidin Blue staining solution
Hepatogenesis	Albumin antibody Staining
Neurogenesis	MAP2 antibody Staining
Osteogenesis	Alizarin Red S

Tab. 2

(c) Primers and antibodies

RT-PCR Primers

	Sequence Forward	Sequence Reverse	band size [in bp]	Tm
GAPDH	5'-CGAGATCCCTCCAAAATCAA-3'	5'-GGTGCTAAGCAGTTGGTGGT-3'	239	
CD4	5′-CTCCCCACTGCTCATTTGGAT-3′	5′-AACAGTCCCATGCTCCATGCT-3′	102	60°C
CD8	5'-TTTCGGCGAGATACGTCTAACCCTGTGC-3'	5'-TTTAGCCTCCCCCTTTGTAAAACGGGCG-3'	379	60°C
CD14	5'-GCCCTTACCAGCCTAGACCT-3'	5'-CCCGTCCAGTGTCAGGTTAT-3'	404	60°C
CD32	5' –CCTCACCTGGAGTTCCAGGAGGGAG- 3'	5' – TAGATCAAGGCCACTACAGCAGCAA- 3'	334	60°C
CD64	5'-ACACCACAAAGGCAGTGA-3‘	5'-CACCCAGAGAACAGTGTT-3‘	881	60°C
CD68	5‘-GCTACATGGCGGTGGAGTACAA-3‘	5‘-ATGATGAGAGGCAGCAAGATGG-3‘	263	60°C
CCR5	5'-CAAAAAGAAGGTCTTCATTACACC-3'	5'-CCTGTGCCTCTTCTTCTCATTTCG-3'	189	60°C
CSFR1	5' -GCCTGTCTCCACTTCTTCAA-3´	5' -GGTATCCATCCTTCACCAGT-3´	440	55°C
IFNGR1	5‘-GGCAGCATCGCTTTAAACTC-3‘	5'-GGAGGTGGGGGCTTTTATTA-3'	195	60°C
MCP-1	5′-CAGCCAGATGCAATCAATGC-3′	5′-GTGGTCCATGGAATCCTGAA-3′	198	60°C
MRC1	5'-TGGTTTCCATTGAAAGTGCTGC-3'	5'-TTCCTGGGCTTGACTGACTGTTA-3'	504	55°C

Primers were purchased from Invitrogen

Real-time RT-PCR Primers

	Sequence Forward	Sequence Reverse	band size [in bp]	Tm
GAPDH[1]	5'- GAAGGTGAAGGTCGGAGTC-3'	5'-GAAGATGGTGATGGGATTTC-3'	226	
Hematopoietic markers				
c-KIT[2]	5′-CCGTGGTAGACCATTCTGTG -3′	5′-GTGCCCACTATCCTGGAGTT-3′	195	59°C
PROM1[2]	5'- CCTCTGGTGGGGTATTTCTT-3'	5'-CAGTTTCCGACTCCTTTTGA -3'	210	59°C
CD4[2]	5'- GTAGTAGCCCCTCAGTGCAA-3'	5'-AAAGCTAGCACCACGATGTC -3'	169	58°C
Unspecific macrophage/ monocyte differentiation markers				
CCR5[1]	5' –CAAAAAGAAGGTCTTCATTACACC- 3'	5' – CCTGTGCCTCTTCTTCTCATTTCG- 3'	189	60°C
TNF[2]	5'-TCCTCCAGACACCCTCAACC -3‘	5'-AGGCCCCAGTTTGAATTCTT -3‘	173	60°C
IL-10[2]	5‘-AAGCCTGACCACGCTTTCTA-3‘	5‘-ATGAAGTGGTTGGGGAATGA-3‘	193	60°C
HLA-DRB1[2]	5'- CTGGTGATGCTGGAAATGAC-3'	5'- CAGAAGCCCTTTCTGACTCC-3'	213	59°C
CD14[2]	5' -ACAGGACTTGCACTTTCCAG -3´	5' - TCCAGGATTGTCAGACAGGT-3´	201	58°C
Specific macrophage/ monocyte differentiation markers				
CD68[2]	5‘-CAACTGCCACTCACAGTCCT-3‘	5'- CAATGGTCTCCTTGGAGGTT-3'	159	59°C
CD11b[2]	5′-ACGGATGGAGAAAAGTTTGG-3′	5′- CAAAGATCTCCCGAAGC-3′	232	59°C
MRC1 [2]	5'-GGCGGTGACCTCACAAGTAT -3'	5'- ACGAAGCCATTTGGTAAACG-3'	168	60°C

Primers were purchased from Invitrogen[1] and realtimeprimers.com[2]

Tab. 3

	origin	Final concentration	Company	Catalog no.
1° Antibodies				
Anti-Albumin	Mouse monoclonal IgG2b	0.025 mg/ml	Abcam	ab10241
Anti-CD68	Mouse monoclonal IgG1	0.02 mg/ml	Santa Cruz Biotechnologies	Sc-20060 FITC
Anti-cfms	Rabbit polyclonal	0.01 mg/ml	Chemicon International	CBL776
Anti-MAP2	Mouse monoclonal IgG1	0.02 mg/ml	Abcam	ab11267
Anti-NOS2	Rabbit polyclonal IgG	0.02 mg/ml	Santa Cruz Biotechnologies	Sc-651
2° Antibodies				
FITC	Goat anti mouse	0.002 mg/ml	Invitrogen	T2761
Texas Red	Goat anti rabbit	0.002 mg/ml	Invitrogen	T2767

Tab. 4

(d) Reagents

Item	Company	Cat. No.
1-Monothioglycerol	Sigma, St. Louis, MO, USA	88640
α- Minimum Essential Medium (MEM) 1X	CellGro, Manassas, VA, USA	15-012-CV
β-Glycerophosphate	Sigma, St. Louis, MO, USA	G6376
Agarose, ultra pure	Invitrogen, Carlsbad, CA, USA	15510027
Alizarin Red S	Sigma, St. Louis, MO, USA	A5533
Ascorbate -2 -Phosphate	Sigma, St. Louis, MO, USA	A8960
Butylated hydroxyanisole (BHA)	Sigma, St. Louis, MO, USA	B1253
Collagenase Type I	Invitrogen, Carlsbad, CA, USA	17100017
Dexamethasone	Sigma, St. Louis, MO, USA	D4902
Dulbecco's Modification of Eagle's Medium (DMEM)	Cellgro, Herndon, VA, USA	10-013
Dimethyl Sulfoxide (DMSO)	Sigma, St. Louis, MO, USA	D2650
Ethanol, anhydrous	Thermo Fisher Scientific, Waltham, MA, USA	A405P-4
Ethidium Bromide	Sigma, St. Louis, MO, USA	E1510
Fetal Bovine Serum (FBS)	Atlanta Biologicals Atlanta, GA	511550
Formaldehyde	Sigma, St. Louis, MO, USA	F8775
Forskolin	Sigma, St. Louis, MO, USA	F3917
L-Glutamine 1%	Cellgro, Herndon, VA, USA	25-005-CI
Hydrocortisone	Sigma, St. Louis, MO, USA	H0888
Indomethacin	Sigma, St. Louis, MO, USA	I7378
Isobutylmethylxanthine	Sigma, St. Louis, MO, USA	I5879
Insulin	Sigma, St. Louis, MO, USA	I9278
KCl	Sigma, St. Louis, MO, USA	P5405
Oil Red O solution	Diagnostic Biosystems, Pleasanton, CA, USA	KT 025-IFU
Para-formaldehyde	Sigma, St. Louis, MO, USA	476081LFF
Phosphate- Buffered Saline (PBS) 10X	CellGro, Manassas, VA, USA	46-013-CM
Penicillin- Streptomycin Solution	Cellgro, Herndon, VA, USA	30-002-CI
Reagent Alcohol	Sigma, St. Louis, MO, USA	R8382-1GA
Rnase ERASE	ICN Biomedicals	821682
Tris-Acetate-EDTA buffer (TAE) 10X	Cellgro, Herndon, VA, USA	46-010-CM
Toluidine Blue	Diagnostic Biosystems, Pleasanton, CA, USA	KT 019-IFU
Triton X100 (TX-100)	Sigma, St. Louis, MO, USA	234729
Trypan Blue	Sigma, St. Louis, MO, USA	T8154
Trypsin 0.5%/ EDTA 0.2%	Cellgro, Herndon, VA, USA	25-052-CI
Valproic Acid	Sigma, St. Louis, MO, USA	P4543
Water, Molecular Biology Grade	Cellgro, Herndon, VA, USA	46-000-CI

(e) Assays and Kits

Item	Company	Cat. No.
100 bp DNA ladder	New England BioLabs, Ipswich, MA, USA	N0467S
α-Naphthyl Acetate Esterase Kit	Sigma, St. Louis, MO, USA	91A-1KT
AlexaFluor labeled Escherichia coli beads	Molecular Probes, Carlsbad, CA, USA	E-13231
GoTaq® Green Master Mix Kit	Promega, Madison, WI, USA	M7122
High Capacity Reverse Transcription Kit	Applied Biosystems, Foster City, CA, USA	4374966
HLTV III_B	AIDS Research & Reference reagent program, Bethesda, MD, USA	
IL-1ß	Sigma, St. Louis, MO, USA	I9401
IL-3	Prospec Bio, Rehovot, Israel	CYT-210
IL-4	Pepro Tech, Inc.,Rocky Hill, NJ, USA	AF-200-04
Lipoplysaccharide (LPS) from E. coli 0127:B8 1mg/ml	Sigma, St. Louis, MO, USA	L5668-2ML
M-CSF	Sigma, St. Louis, MO, USA	M 6518
p24 ELISA Kit	ZeptoMetrix, Buffalo, NY, USA	
RNeasy mini Kit	Qiagen, Valencia, CA, USA	74106
SYBR Green Master Mix	Invitrogen, Carlsbad, CA, USA	QR0100
rhHGF	R&D Systems, Minneapolis, MN, USA	294HG
rhOSM	R&D Systems, Minneapolis, MN, USA	295OM
StemPro Chondrogenesis Differentiation Kit	GIBCO, Invitrogen, Carlsbad, CA, USA	A10071-01
Q-Plex™ Human Cytokine Array - Screen	Quansys Biosciences, West Logan, UT, USA	110251HU

(f) Equipment

Item	Company
6, 12, 24, 48 Well Plates	Greiner Bio-One, St. Louis, MI, USA
96 Well Flat Bottom Plates	Greiner Bio-One, St. Louis, MI, USA
96 Well Plates, ultra low adhesion	Greiner Bio-One, St. Louis, MI, USA
96 Well PCR Plates	BioRad Laboratories, Hercules, CA, USA
Bottle Top Filters, 0.22µm	Corning Inc., Lowell, MA, USA
Cell Strainers, 40µm 100µm	BD Biosciences, San Jose, CA, USA
Chamber Slides	Nalgene Nunc , Rochester, NY, USA
Conical Tubes, 15ml	Greiner Bio-One, St. Louis, MI, USA
Conical Tubes, 50ml	Greiner Bio-One, St. Louis, MI, USA

Cover Slips, Glass, 12mm	Ted Pella, Redding, CA, USA
Cover Slips, Plastic, 13mm	Nalgene Nunc , Rochester, NY, USA
Cryotubes	Greiner Bio-One, St. Louis, MI, USA
Disposable Vacuum Filtration System	Millipore, Billerica, MA, USA
Eppendorf Tubes	Greiner Bio-One, St. Louis, MI, USA
Filter Units	Nalgene Nunc , Rochester, NY, USA
Microscope Cover Slips	Ted Pella, Redding, CA, USA
Microscope Slides charged/ non-charged	Ted Pella, Redding, CA, USA
Nitrile Gloves	Thermo Fisher Scientific, Waltham, MA, USA
Opticell Tips & Culture Chambers	Nalgene Nunc , Rochester, NY, USA
Petri Dishes	Greiner Bio-One, St. Louis, MI, USA
Pipette Tips filtered/ non-filtered 0.1-20µl, 1-20µl, 1-200µl, 10-100µl, 101-1000µl	USA Scientific Inc., Ocala, FL, USA
Polystyrene Falcon Round-Bottom Tubes, 5ml	BD Biosciences, San Jose, CA, USA
T-25, 75, 175 Cell Culure Flask	Greiner Bio-One, St. Louis, MI, USA
Volumetric Pipettes 1, 2, 5, 10, 25, 50 ml	Greiner Bio-One, St. Louis, MI, USA
Repeater Pipettes	Thermo Fisher Scientific, Waltham, MA, USA
Pasteur Pipettes	Thermo Fisher Scientific, Waltham, MA, USA
Scalpels	FEATHER, Japan
Transfer Pipettes	Thermo Fisher Scientific, Waltham, MA, USA

Item	**Company**
ChemiDoc™ XRS+ System	BioRad Laboratories, Hercules, CA, USA
Conical Tube Shaker	Thermo Fisher Scientific, Waltham, MA, USA
CCD imaging system, FluoChem 8900	Cell Biosciences, Santa Clara, CA, USA
Eppendorf Master Cycler	Eppendorf, Westbury, NY, USA
Exposure Controller	Zeiss, Germany
Flow Cytometer, Epics FC500	Beckman Coulter, Fullerton, CA, USA
Gel Electrophoresis System	BioRad Laboratories, Hercules, CA, USA
Incubator Equatherm	Lab-Line
Lambda DG-4	Sutter Instrument Company, Novato, CA, USA
Micropipettor 2,10, 20, 100, 200, 1000	Socorex, Switzerland
Micropipettor 12-channel 20	Socorex, Switzerland
Microscope Eclipse TE2000-U	Nikon, Japan
Microscope, TCS SP-2 confocal	Leica, Germany
Nanodrop spectrophotometer ND-1000	Thermo Fisher Scientific, Waltham, MA, USA
Pipette Controller Accu-Jet	BrandTech Scientific, Essex, CT, USA
Real-Time PCR System iCycler My iQ	BioRad Laboratories, Hercules, CA, USA
RS Photometrics Cool Snap camera	Photometrics, Tucson, AZ, USA
Sterile Hood	
Temperature Regulated Centrifuge	IEC
Water bath	Thermo Fisher Scientific, Waltham, MA, USA
Water Jacketed Incubator	Thermo Fisher Scientific, Waltham, MA, USA

(g) Software

Item	Company
CXP software	Beckman Coulter, Fullerton, CA, USA
iQ5 V2.0 software	BioRad Laboratories, Hercules, CA, USA
Leica confocal software	Leica, Germany
MetaMorph V6.1 Imaging Software	Universal *Imaging* Corp., Downington, PA, USA
Microsoft Office Excel 2007 software	Microsoft Corp., Redmond, WA, USA
ModFit LT V3.1 software	Verity Software House, Topsham, ME, USA
NanoDrop 1000 3.7.0 software	Thermo Fisher Scientific, Waltham, MA, USA
Q-ViewTM Plus array software	Quansys Biosciences, West Logan, UT, USA
1.1.1 Quantity One 1-D Analysis Software	BioRad Laboratories, Hercules, CA, USA
SPSS	SPSS Science, Chicago, IL, USA

Tab. 5

Methods

ASC Isolation and Culturing

The human ASCs were isolated from subcutaneous adipose tissue of healthy donors* based on standard protocols (Image 1); fat tissue was provided by Dept. of Surgery, Tulane University Health Science Center, NO, LA. The collection of all human tissue samples was approved by Institutional Review Board (IRB) of Tulane University, School of Medicine, New Orleans, Louisiana.

First, 50g of cohesive pieces of adipose tissue were washed intensely in a petri desk with 10ml of PBS supplemented with 2% Penicillin/ Streptomycin followed by mechanical comminution by using a steril scalpel and tissue forceps. Then, the finely minced adipose tissue was incubated in 20ml of collagenase I for 45 minutes at 37°C as previously described [27]. The enzymatically digested tissue was sequentially filtered through a cell strainer (0.22µm pore size) and centrifuged at 1200*x* g for 4 minutes. The supernatant containing adipocytes and debris was discarded, the pelleted cells were washed twice with PBS and finally were resuspended in growth media and plated on plastic cell culture ware at a fixed density at 37°C in 5% CO_2. Growth media contained a-modification of Eagle's medium (α-MEM), 20% FBS (heat inactivated), 1% L-Glutamine, 1% Penicillin/ Streptomycin (1:1) and was regularly changed in the following. The cell cultures were passaged at a confluency of ~70% using 0.5% trypsin plus 0.2% EDTA; cell viability was intermittently controlled with trypan blue staining to be greater than 95% throughout the entire term.

ASC Clone Generation

For ASC clone generation, ASCs at passage 2 were counted with a hemocytometer and cultured on ultra low adhesion 96-well plates at a density of a single cell per well, which was confirmed by microscopy. Wells containing either none or more than one cell were excluded from further analysis. Clones were then cultured and expanded in maintenance media.

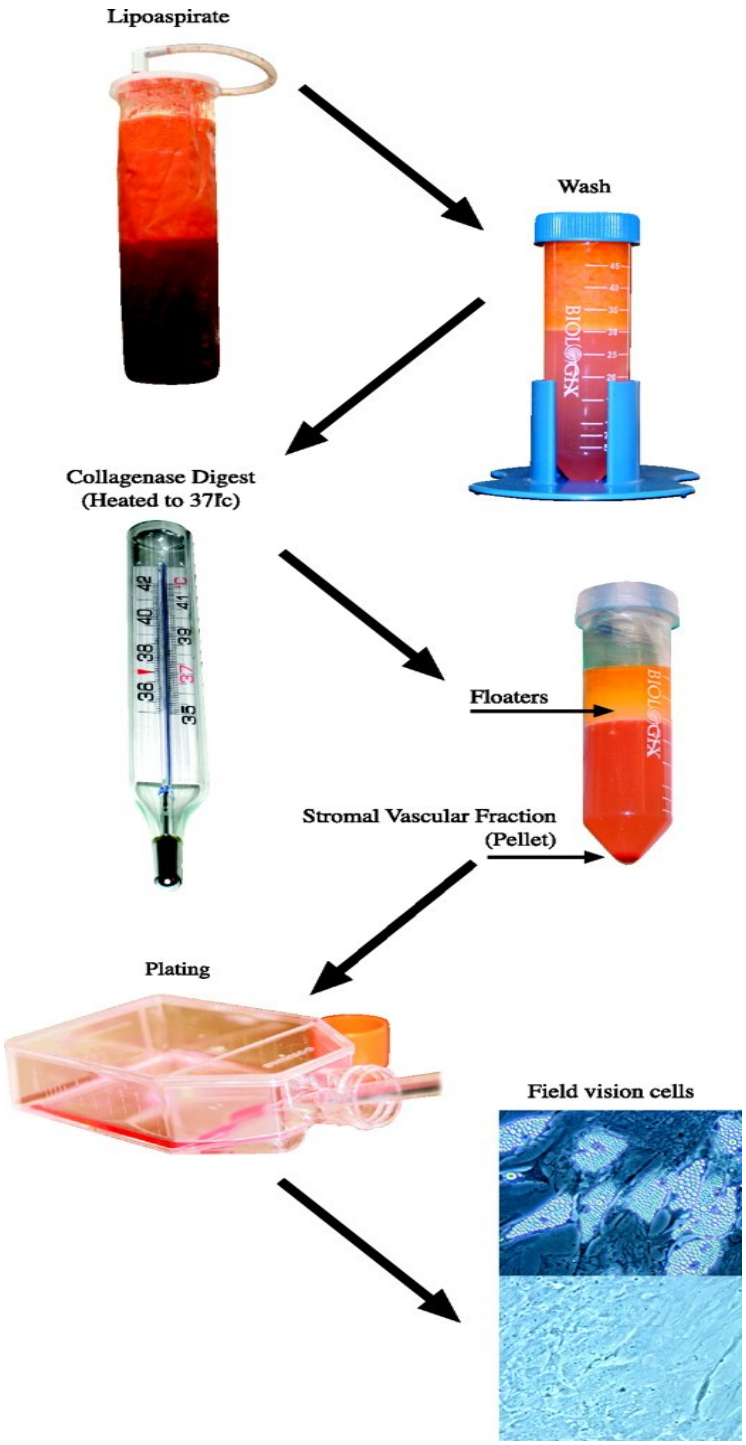


Image 1 Isolation of ASCs. Fat tissue is minced followed by PBS washing and subsequent enzymatic digestion with collagenase I for 45min at 37°C. Pelleted cells represent the SVF and get cultured in maintenance medium (passage 0). Instead of liposuction cohesive pieces of fat tissue were used. Image source[14]

Multililineage Differentiation Assays

The multilineage potential of clonal ASCs was examined by performing adipogenic, chondrogenic, osteogenic, hepatogenic¤, and neurogenic¤ differentiation assays as previously described [28-30]. ASCs at low passages were placed in the accordant differentiation media (Tab.1). The specific differentiation media was changed every 2 days over a time period of 14 days.

The adipogenic cultures were incubated in an α-MEM based differentiation media supplemented with i.a. dexamethasone, isobutylmethylxanthine and indomethacin and subsequently stained with Oil Red O solution. The osteogenic differentiation media consists i.a. dexamethasone, ascorbate-2-phosphate and β-glycerophosphate. The differentiated cultures were stained with Alizarin Red. For chondrogenesis the StemPro Chondrogenesis Differentiation Kit from GIBCO was used, and Toluidin Blue staining confirmed chondrogenesis. Hepatogenic and neurogenic differentiation were induced and verified by albumin and MAP2 antibody staining respectively (Tab. 2).

¤Hepatogenic and neurogenic differentiation assays were performed in collaboration with X. Bai, Department of Molecular Pathology, University of Texas MD Anderson Cancer Center, TX USA.

Hematopoietic differentiation

1. Development of hematopoietic differentiation media

It is well known that hematopoietic differentiation is induced by a varied spectrum of colony-stimulating factors and chemokines. In order to develop a specific media to differentiate ASCs towards macrophages, an extensive investigation of published protocols for diverse types of stem cells and progenitor cells has been carried out [22,31-36]. Comparative analysis of the used components revealed the importance of GM-CSF, M-CSF, IL-3 for the induction of myeloid characteristics. IL-1β as an intense pro- inflammatory cytokine is very likely to support further maturation and activation [37]. 1-MTG has been used as reducing agent in order to promote differentiation [35]. Just as the maintenance media for undifferentiated ASCs, the differentiation media is based on α-MEM, supplemented with FBS and therefore provides the requirement for cell growth and development.

In the following, the exact concentrations of the components have been investigated by multiple trials under various configurations (Tab. 6a,b). Hereunder, the cultures were evaluated for confluency, cell growth, morphology and adherence by microscopy and additionally for cell viability analysis using Trypan Blue staining. On the basis of these criteria the optimal composition of the differentiation media could be identified:

First, ASCs were cultured under conditions based on maintenance media (α-MEM + 20% FBS). To induce differentiation, the stem cells were exposed to 20U/ml M-CSF and IL-3 in increasing concentrations. Under low concentrations of IL-3, no significant difference in comparison to the control could be observed, whereas higher concentrations of IL-3 in combination with M-CSF lead to a beginning alteration of the morphology, decreased adherence and doubling time indicating a reduction of stem cell characteristics. Stable morphological alteration combined with high cell viability could be found upon 500U/ml IL-3 when supplemented with 20U/ml M-CSF. The additional use of IL-1β (Tab. 6a) and 10ng/ml MTG (Tab 6b) preponed this phenomenon. Higher concentrations of MTG as well as the absence of FBS involved decreased cell viability.

For optimal hematopoietic differentiation, the devised α-MEM based media consists of 10% FBS, supplemented with 0.1 µl/ml 1-MTG, 100 U/ml IL-1β, 500 U/ml IL-3 and 20 U/ml M-CSF.

Setting 1:

in α-MEM + 20% FBS + 0nl/ml MTG					
	Control ASCs	ASCs + 20U/ml M-CSF			
	0U/ml IL-3	0U/ml IL-3	250U/ml IL-3	500U/ml IL-3	750U/ml IL3
0U/ml IL-1β	○	○	○	(x)	(x)
50U/ml IL-1 β	○	○	(x)	x	x
100U/ml IL-1 β	○	○	(x)	x	x
150U/ml IL-1 β	○	○	(x)	x	x

Tab. 6a Control ASCs and ASCs undergoing differentiation were plated in α-MEM + 20% FBS. To promote hematopoiesis, cells were induced with 20U/ml M-CSF and IL-3 in increasing concentrations. An IL-1β gradient was applied to both arms. Best results were obtained with at least 100U/ml IL-1β and 500U/ml IL-3. Higher chemokine rates did not lead to significantly higher differentiation potential.
○ no differentiation (x) beginning differentiation **x** stable evidence for differentiation † cell death

Setting 2:

Control ASCs				**ASCs + 20U/ml M-CSF , 500U/ml IL-3, 100U/ml IL-1 β**		
	0nl/ml MTG	10nl/ml MTG	30nl/ml MTG	0nl/ml MTG	10nl/ml MTG	30nl/ml MTG
0% FBS	†	†	†	†	†	†
10% FBS	○	○	†	(x)	x	†
20% FBS	○	○	†	(x)	x	†

Tab. 6b Differentiation was conducted under stabile conditions with 20U/ml M-CSF, 500U/ml IL-3 and 100U/ml IL-1β. Both, control and differentiating ASCs, were exposed to increasing concentrations of MTG and FBS, revealing optimal results at 10%FBS and 10nl/ml MTG.
○ no differentiation (x) beginning differentiation **x** stable evidence for differentiation † cell death

2. Cell culture protocol for hematopoietic differentiation

ASC clones were plated at a density of 5,000 cells/cm^2 on either cell culture dishes or chamber slides. Cells were cultured in differentiation media consisting of α-MEM, 10% FBS, 0.1ml/ml 1-Monothioglycerol (MTG), supplemented with 100U/ml IL-1β, 500U/ml IL-3, and 20U/ml M-CSF as stimulating substances. Thirty percent of the primary volume was augmented with fresh differentiation media every 2 days for 12 days. Cultures of ASC clones in growth media containing 10% FBS served as undifferentiated control.

Morphological Analysis

To document morphological changes during hematopoietic differentiation, HD and ASC control cultures were observed with a Nikon Eclipse TE 2000-U microscope and photographed using an RS Photometrics Cool Snap camera; data was analyzed with MetaMorph V6.1 software.

Cell Cycle Analysis

For cell cycle analysis 10^6 cells per sample were fixed with 70% EtOH for 2h and subsequently incubated in 1ml PI/ TX-100 staining solution with RNase A (10ml 0.1% TX-100 in PBS + 2mg DNase free RNase + 200µl of 1mg/ml PI) for 30min at RT.
Cell cycle analysis was performed at the Cell Analysis Core Facility, Tulane Cancer Center (Tulane University Health Science Center, New Orleans LA.); data analysis was carried out using ModFit LT V3.1 software.

Flow cytometry

Flow cytometry was performed on trypsin-dissociated cell cultures that were immunostained for CD105, CD90, CD44, CD34, CD45, CD4, CD11b, and CD68, followed by three rinsing cycles with PBS. The fluorescent signal was detected by a Beckman-Coulter Epics FC500 flow cytometer; data was carried out with CXP software.

Reverse transcription-PCR

Total cellular RNA was isolated from ASC cultures using an RNeasy mini kit. The High Capacity Reverse Transcription Kit was used to obtain the cDNA. Nucleic acid concentrations were photometric measured (Nanodrop spectrophpotometer ND-1000). For RT-PCR, GoTaq® Green Master Mix was applied on 500ng cDNA per reaction tube. Thermocycling was performed at 95°C for 3 min, followed by 34 cycles at 94°C for 45 sec, Tm for 45 sec, 72°C for 60 sec completed by additional 10 min 72°C (Tab. 3). Human GAPDH was served as housekeeping gene□, a non template control was used to exclude contamination.

The PCR product was confirmed by gel electrophoresis (2% ultra pure agarose in 1xTAE buffer), including a 100bp marker as DNA molecular weight marker.

Gels were analyzed with an ultraviolet transilluminator gel imaging system (ChemiDoc™ XRS) and semi- quantitative data was carried out by Quantity One 1-D Analysis Software.

Real-time reverse transcription-PCR

Real-time PCR was performed using SYBR Green Master Mix in a two-step protocol (40 cycles of 10 sec at 95°C and 35 sec at Tm). The primers were selected based on the linearity of the threshold cycle (CT) values obtained in the serial dilutions of the template, with the negative controls containing no template. Annealing curves were performed to ensure the absence of primer dimers. The data were generated with an iCycler My iQ and analyzed using iQ5 V2.0 software. The relative gene expression levels were quantified based on the CT, and normalized to the reference gene GAPDH. Each reaction was run in triplicate with the results averaged. The data are representative of the means of three independent experiments on each clone. The fold change for each gene expression was calculated based on the equation $2^{-\Delta\Delta CT}$.

□housekeeping gene = constitutive gene, expressed in all cells at high levels; e.g. GAPDH [38]

Immunocytochemistry

Cultures of ASC clones were differentiated on chamber slides. For fixation and permeabilization, the cell cultures were carefully rinsed twice in PBS before set in 1% para-formaldehyde for 10 min, followed by 15 min in 0.05% Triton-X 100 in PBS and 10 min in 4% para- formaldehyde. Completing with a final washing step the slides were incubated with human specific primary antibodies for CD68, CSF1R, and NOS2 at a final concentration of 0.02–0.04 mg/ml, then incubated with 0.002 mg/ml of the matching secondary antibody (Tab. 4). The signal was detected with a Leica TCS SP-2 confocal microscope equipped with one Argon (457–477 nm; 488 nm, 514 nm) and two HeNe lasers (543 nm; 633 nm) at a magnification of HCX PL APO 63x/1.4 at 21°C. Data was processed with Leica confocal software. HSC slides were prepared by cytospin centrifugation (400 rpm for 8 min) for immunostaining and served as positive control.

Functional assays

Non-specific esterase staining

Non-specific esterase activity was determined by using the α-Naphthyl Acetate Esterase Kit according to the manufacturer's instructions. Briefly, cells were fixed and stained with α-naphthyl acetate solution for 30 min at 37°C then counterstained with Hematoxylin Solution and were evaluated microscopically. When enzymatically hydrolyzed, α-Naphthyl acetate liberates free naphthol, which couples with diazonium compound and forms dark colored deposits at sites of non-specific esterase activity. The result was interpreted based on HSC staining (positive control) or undifferentiated ASCs (negative control).

Assay for phagocytosis

The phagocytotic ability of differentiated cells was determined by incubating the HD cells with Alexa Fluor labeled Escherichia coli beads at a final concentration of 100 particles/ cell for 2h. Cells were thoroughly rinsed twice with PBS and imaged using a Leica TCS SP-2 confocal microscope.

IL-4 and LPS stimulation

The differentiated cells as well as the control culture were activated with 20ng/ml IL-4 for 5 days or with 1µg/ml lipoplysaccharide (LPS) from E. coli O127:B8 for 3 days. Additionally, supernatants from the cultures were harvested after 1h, 2h, 5h, 8h, 24h, 48h, 3 days and 5 days and frozen at -20°C.

ELISA of inflammatory cytokines

The ELISA assay was performed using Q-Plex™ Human Cytokine Array to screen the supernatants of the stimulation assays for various cytokines (IL-1α, IL-2, IL-5, IL-10, IL-13, IL-17, IFNγ, TNFα, and TNFβ). The chemiluminescent signal was detected using a CCD imaging system (Alpha Innotek FluoChem 8900) and finally analyzed with the Quansys Q-View^{TM} Plus array software.

HIV-1 infection

Cells were exposed to cell-free HIV-1 at titers of ~100 pg/ml of p24 [103–104 TU/ml] for 24h. HIV-1 was obtained from supernatants of HTLV-III$_B$ (AIDS Research & Reference Reagent Program, Bethesda, MD) infected HUT78 T4-lymphocytes. HD cells were infected on either five (5-HD) or eight (8-HD) days post induction of differentiation. Undifferentiated ASCs served as negative, HUT78 cells as positive control and were infected under the same conditions as described above. After 24h exposure time, cell cultures were washed repeatedly with fresh media to remove unattached viral particles before cultured again for either 3 or 5 days.

Viral p24 levels were analyzed by ELISA to monitor the viral replication in undifferentiated, HD, and HUT78 cells.

Statistics

Data are presented as mean ±SD in each figure. Comparisons between values were evaluated using analysis of variance (ANOVA) or t-test. Differences were considered significant at $P \leq 0.05$.

Results

Derivation of macrophages from ASCs

To obtain a pure multipotent mesenchymal stem cell population, a single-cell clonogenic isolation technique was employed, in which, ASCs obtained from adipose tissue of healthy donors (n=43; age 17–33 years, mean=27.6) were plated at a density of one cell per well in serum free medium in 96 well cell culture dishes. The culture dishes were inspected on day 1 to confirm the presence of one cell per well. A total of 19 clones were available within 7 days. Seven out of 19 clones failed to grow in the serum free medium, and the remaining 12 viable clones were continuously cultured to confluency, and expanded for further characterization.

Immunophenotyping confirmed that the adherent ASC clones displayed mesenchymal markers such as $CD90^{+}$, $CD105^{+}$, $CD44^{+}$, $CD4^{-}$, $CD11b^{-}$, $CD34^{-}$, $CD45^{-}$, and $CD68^{-}$, indicating the absence of any hematopoietic progenitor cells (Fig. 1a). All clones had equivalent colony formation potential and displayed the capability of differentiating into adipogenic, osteogenic, chondrogenic, hepatogenic, and neurogenic lineages *in vitro* (Fig.1b).

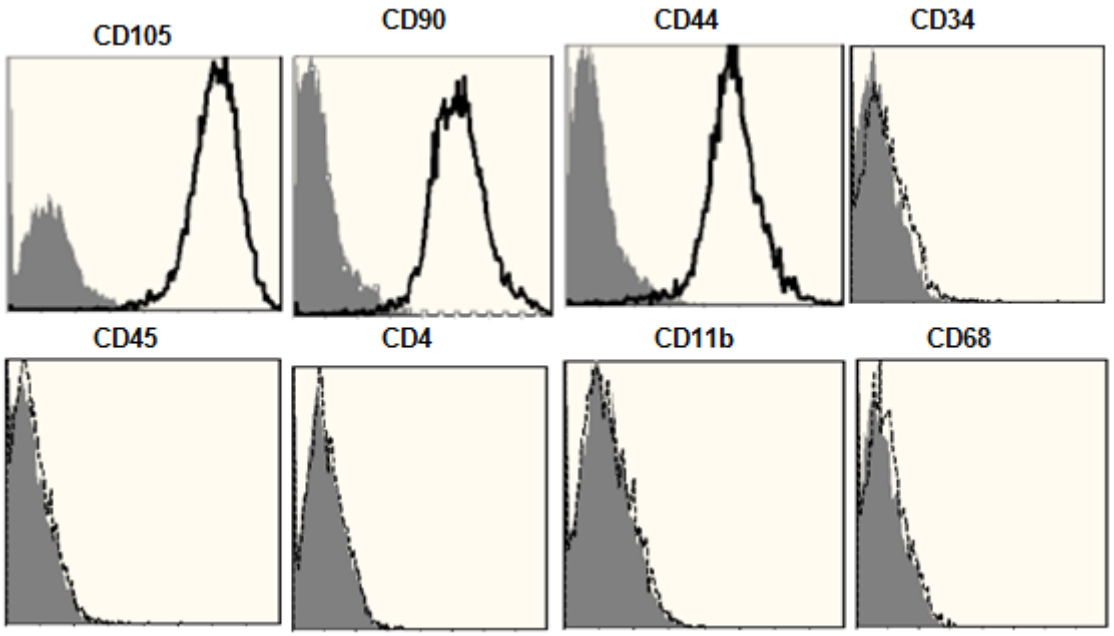


Fig.1a Immunophenotypic analyses of cell surface profile of ASC clones (dashed line) including isotype staining (filled histogram). Clonally expanded cells were stained with monoclonal antibodies for CD105, CD90, CD44, CD34, CD45, CD4, CD11b, and CD68. Flow cytometry histographs are representative of triplicate experiments of each clone.

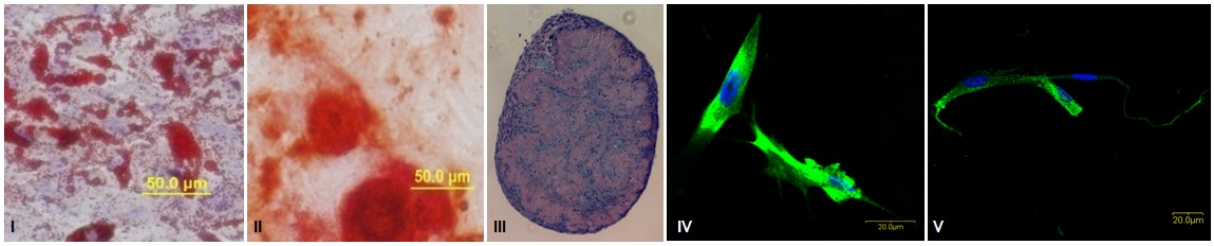


Fig.1b Multilineage differentiation potential of ASC clones. Clonally isolated cells maintained their stem cell characteristics and were capable of differentiating into (I) adipogenic (differentiated cells were stained positive for intracellular lipid vesicles using Oil Red O), (II) osteogenic (differentiated cells were stained with alizarin red), (III) chondrogenic (extracellular proteoglycans were stained with toluidin blue), (IV)¤ hepatogenic (differentiated cells were stained for the expression of albumin), and (V)¤ neurogenic lineages (immunohistochemistry indicated theexpressionofMAP2ondifferentiated cells). ¤ *Images were provided by X.Bai, Dept. of Mol. Pathology, Univ. of Texas MDACC, TX USA*

For hematopoietic differentiation, ASC clones were cultured in a differentiation media consisting of IL-1ß, IL-3, macrophage colony stimulating factor (M-CSF), and MTG. Incubation of ASCs in this cocktail of proinflammatory cytokines resulted in a significant alteration in cell morphology from spindle to round shaped cells within 9 days (Fig. 1c). As the differentiation proceeded (12 to 15 days) cells lost their adherence capabilities. These observations were consistent in identical experiments on 12 ASC clones. The cell growth and proliferation ceased upon the initiation of morphological change into hematopoietic differentiated (HD) cells. Control cultures showed no morphologic alteration. Cell cycle analysis of the HD cells showed an arrest in the S-phase indicating a cessation of cell proliferation (0.00%) compared to 3.35% in the control. However the G1/G2 ratio remained constant at 1.90. However, the entire population remained diploid at 15 days of differentiation. (Fig. 1d)

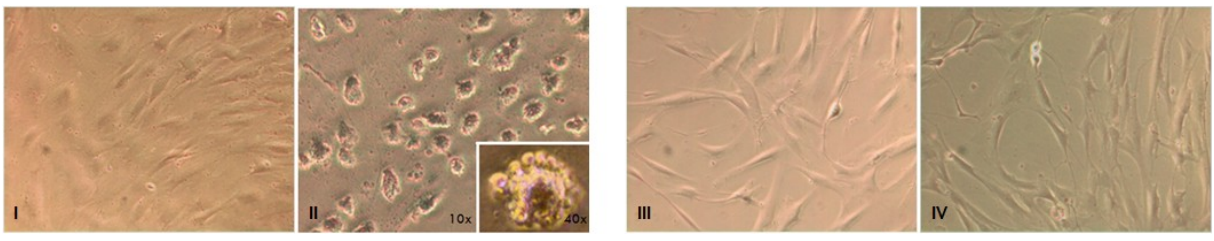


Fig.1c For hematopoietic differentiation, ASC clones were cultured in differentiation media. The morphology of cells altered from day 3 (I) to day 9 (II) of differentiation. Cells cultured in control media (α-MEM +10% FBS +0.1µl/ml MTG) exhibit no change in morphology or in adherence after (III) 3 days and (IV) 9 days. Images were taken with a Nikon Eclipse TE2000-U microscope at a magnification of 10x/0.3 and 40x/0.6 captured by an RS Photometrics Cool Snap camera at 21°C.

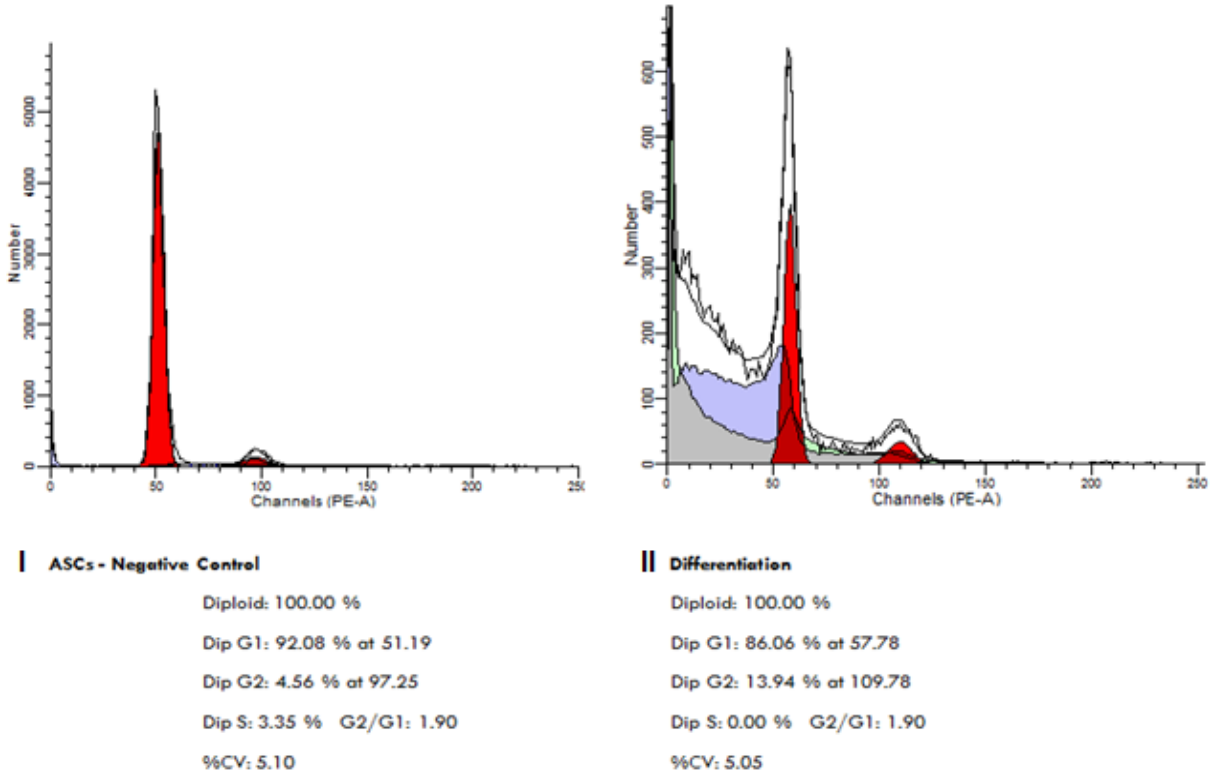


Fig.1d Cell cycle analysis in ASCs shows 92.08% of the cells in G1 phase, 4.56% in G2 phase and an S phase of 3.35%. 100% of the cells are diploid (I). After 12 days of hematopoietic differentiation towards the hematopoietic lineage, the S phase drops down to 0%, showing that the cells do not replicate any more (II). Supporting observations in the culture of a slower growth and an increasing cell death the cell cycle analysis shows a shift from a decreasing number of cells in G1 phase to an increasing percentage in G2 phase (86.06% and 13.94% respectively). However, the ratio G2/ G1 remains stable at 1.90 and the differentiated cells show a diploid karyotype throughout the entire term.

HD cells were assessed for the expression of lineage specific genes indicating the level of differentiation and maturation, including hematopoietic markers (c-KIT, PROM1, CD4), early monocyte/ macrophage markers (IFNGR1, CCR5, HLA-DRB1, TNF, IL10, CD14), and mature monocyte/ macrophage markers (CD68, CD11b, MRC1, CSF1R, CCL2).

Gene expression analysis using semi-quantitative RT-PCR revealed that HD cells express c- KIT and PROM-1, which indicates a genomic shift towards the hematopoietic lineage. Expressions of these genes together with CD4 are known to be associated with hematopoiesis of HSCs in bone marrow. Hematopoietic cells obtained from bone marrow (BMCs) served as positive control in these experiments.

Analyses of early monocyte/macrophage markers revealed a significant up-regulation of CD14 (92.6 O.D.) in the differentiated ASCs comparable to its expression in BM cells (130.28 O.D.). In HD cells CD4 and CD8 gene expression was about 30 percent

of the positive control. CCR5 was marginally higher expressed in HD cells than in BM cells, Interferon γ receptor (IFNGR1; CD119) just slightly less (78%).

Interestingly, a considerable up-regulation of markers of mature monocyte/ macrophages such as colony stimulating factor 1 receptor (CSF1R; CD115), monocyte chemoattractant protein-1 (alias CCL2) and CD68 could be detected in HD cells. These genes were not expressed in undifferentiated ASCs (Fig. 2).

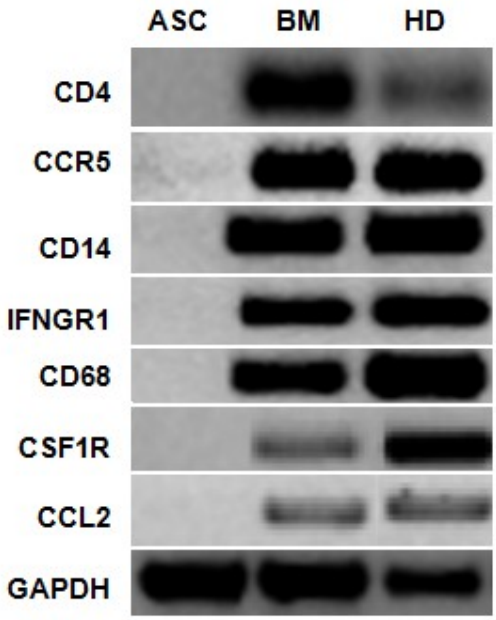


Fig. 2 Semi- quantitative RT-PCR analysis of hematopoetic genes in HD cells. HD cells express genes of hematopoietic lineage (CD4, CD8), early monocyte/ macrophagemarkers (CCR5, CD14, IFNGR1, CD32), and mature monocyte/ macrophage markers (CD68, CSF1R, CCL2). BM cells and undifferentiated ASCs served as positive and negative controls respectively.

Quantitative real-time PCR analysis substantiated these results by showing a significant increase in the expression of the hematopoietic lineage markers CD4, PROM1, and c-KIT (Fig. 3a). mRNA coding for IL-1β and IL-3 receptor genes have been detected in HD cells (2.4±0.6 and 1.8±0.3 respectively; n=3). Similarly, HD cells exhibit an elevation in NFkB expression (2.7±0.4; n=3, $p<0.05$). Compared to undifferentiated ASCs, weak gene expression of GATA and PU.1 (1.08±0.1 for GATA and 1.9±0.3 for PU.1; n=3) was observed in HD cells. Furthermore, there was a considerable up-regulation of early monocyte/macrophage markers such as CD14, major histocompatibility complex class II (HLA DRB1), TNF, CCR5, and IL10 in differentiated cells (Fig. 3b). Among the mature monocyte/ macrophage specific genes, HD cells displayed a strong expression of CD68. In addition, there was an

increase in the expression of integrin α, encoding CD11b (500 fold) and mannose receptor 1 (MRC1; 72 fold) (Fig. 3c).

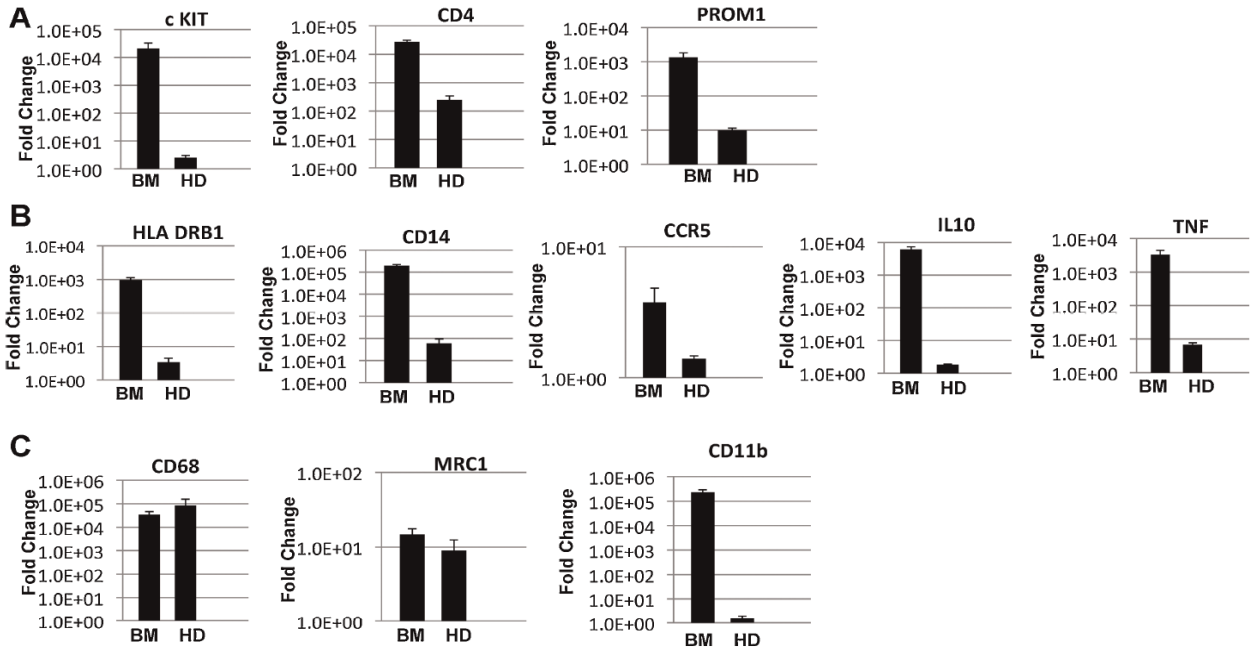


Fig. 3 Quantitative analysis of hematopoietic specific gene expression. (A) ASC clones were incubated for 12 days in differentiation media. Gene expression of hematopoietic markers, (B) markers of early monocyte/ macrophage cells, and (C) mature monocyte/ macrophage markers were analyzed by real-time RT-PCR. Undifferentiated ASC clones were negative for these genes. n=5 for independent differentiation experiments ±SD ($P < 0.05$).

Immunocytochemistry analyses exhibited the expression of hematopoietic lineage specific antigens CD68, CSF1R, and NOS2. Unlike undifferentiated ASCs, all these antigens were readily detectable in HD and BM cells (Fig. 4).

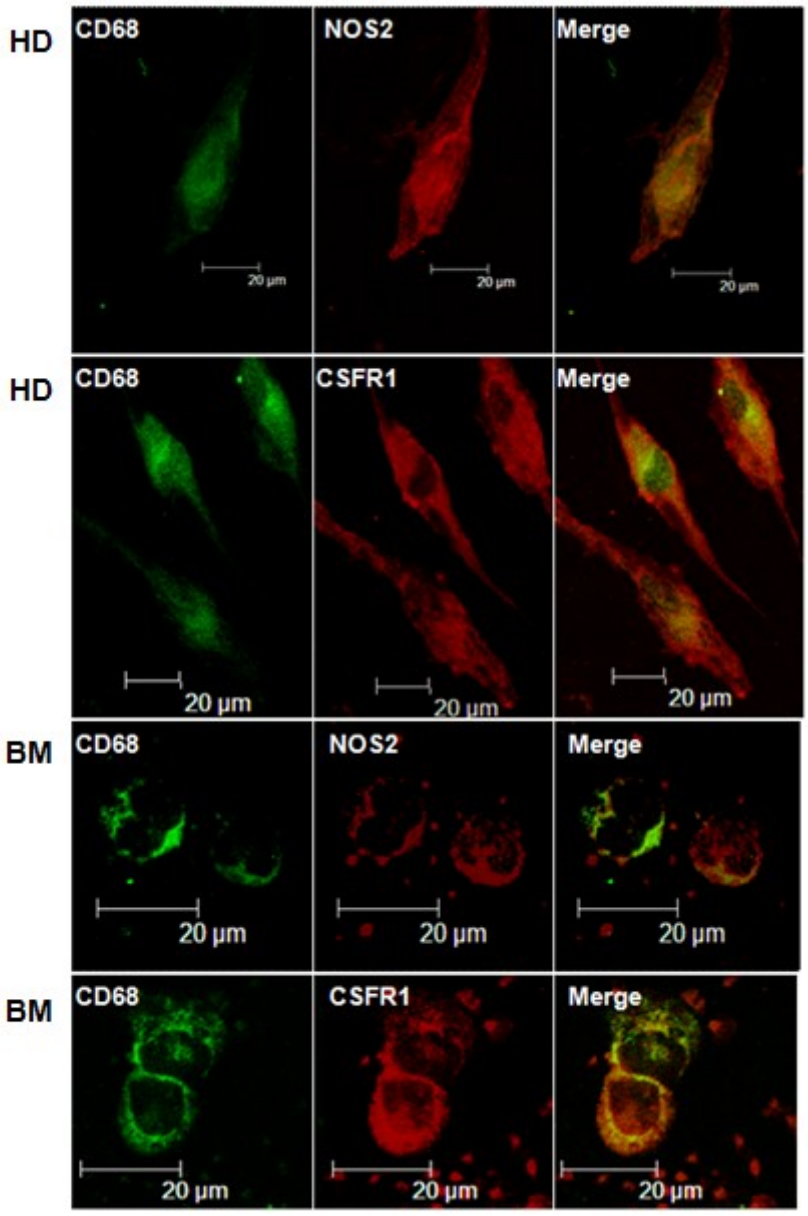


Fig. 4 Immunocytochemistry of HD cells. HD cells were strongly immunoreactive for CD68, CSFR1, and NOS2 following differentiation. BM cells were used as positive control for CD68, CSFR1 and NOS2. Images (representative of triplicate experiments) were taken with a Leica TCS SP-2 confocal microscope at an original magnification of 63x/1.4 oil. Scale bar is 20µm.

HD cells displayed macrophage function

The transcriptomic analysis indicated that HD cells gain characteristics of macrophages. An important function of the macrophage is its ability to phagocytose foreign material. The phagocytic capability of HD cells was evaluated using green fluorescent-labeled *E. coli* particles. Unlike the undifferentiated ASCs, more than 95% of HD cells were able to actively phagocytose the *E. coli* particles (Fig. 5). However, the undifferentiated ASCs did not display any phagocytic activity.

Moreover, similar to monocytes and macrophages [39], HD cells exhibit enzymatic activity of α-Naphthyl acetate esterase (α-NSE) to the extent of about 36.26% of differentiated cells (0.3626 ± 0.0734; n=10) (Fig. 6).

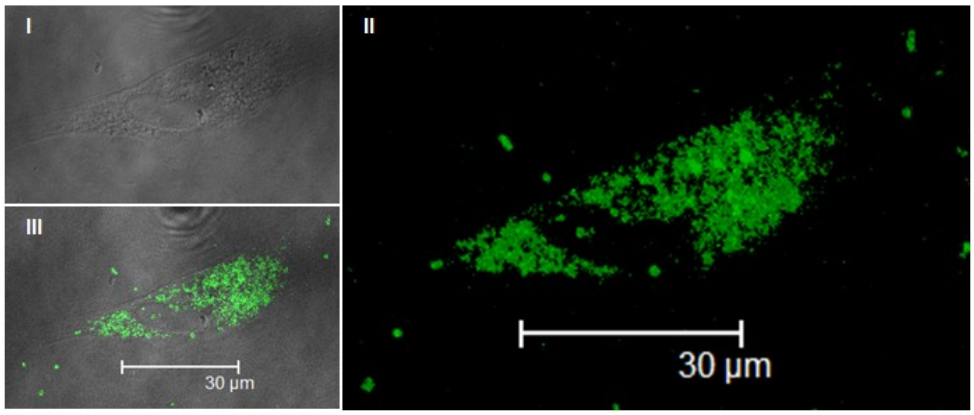


Fig. 5a Confocal microscopic analysis of phagocytic activity of HDcells. Alexa Fluor 488 labeled E.coli beads were phagocytosed by HD cells after 2h of incubation (green), (I) brightfield image, (II) fluorescent signals in HDcells, and (III) an overlay of the images I and II. Images were captured with a Leica TCS SP-2 confocal microscope at a magnification of 63x/1.4 oil at 21-C using Leica confocal software. Undifferentiated ASCs did not show phagocytic activity (Fig. 5c).

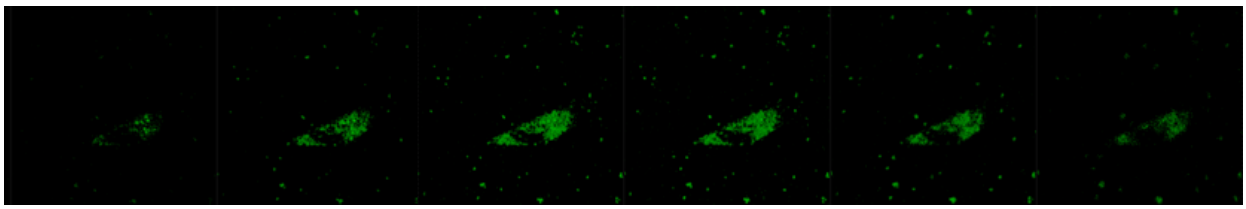


Fig. 5b Image stack along the z-layer determined the location of the fluorescent signal in the cytoplasmic cell compartment. The nucleus remains unstained. Images were taken with a Leica TCS SP-2 confocal microscope at a magnification of 63x/ 1.4 at 21°C using Leica confocal software.

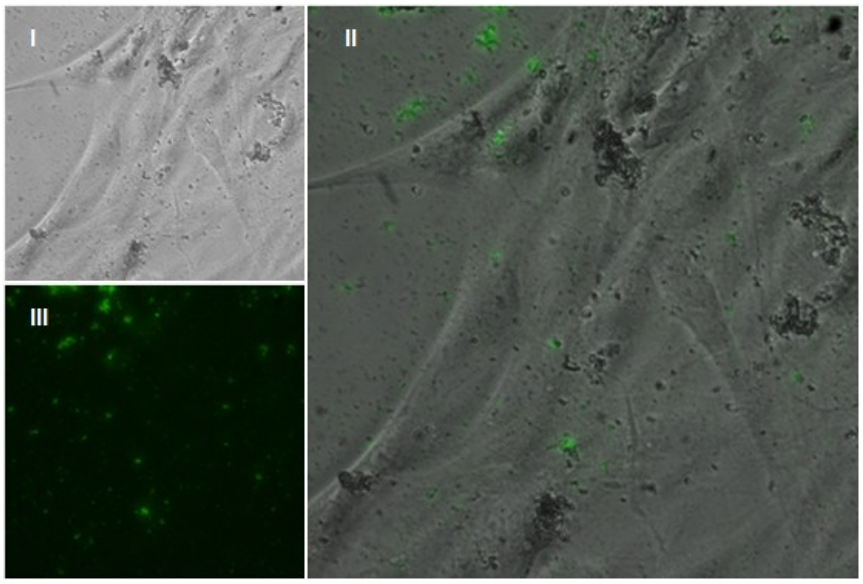


Fig. 5c Undifferentiated cells did not phagocyte Alexa Fluor 488 labeled E.coli beads. After 2h of incubation (I) brightfield image (II) Fluorescent image and (III) overlay of I and II showing no uptake of bacterial particles. Settings are equal to 5a.

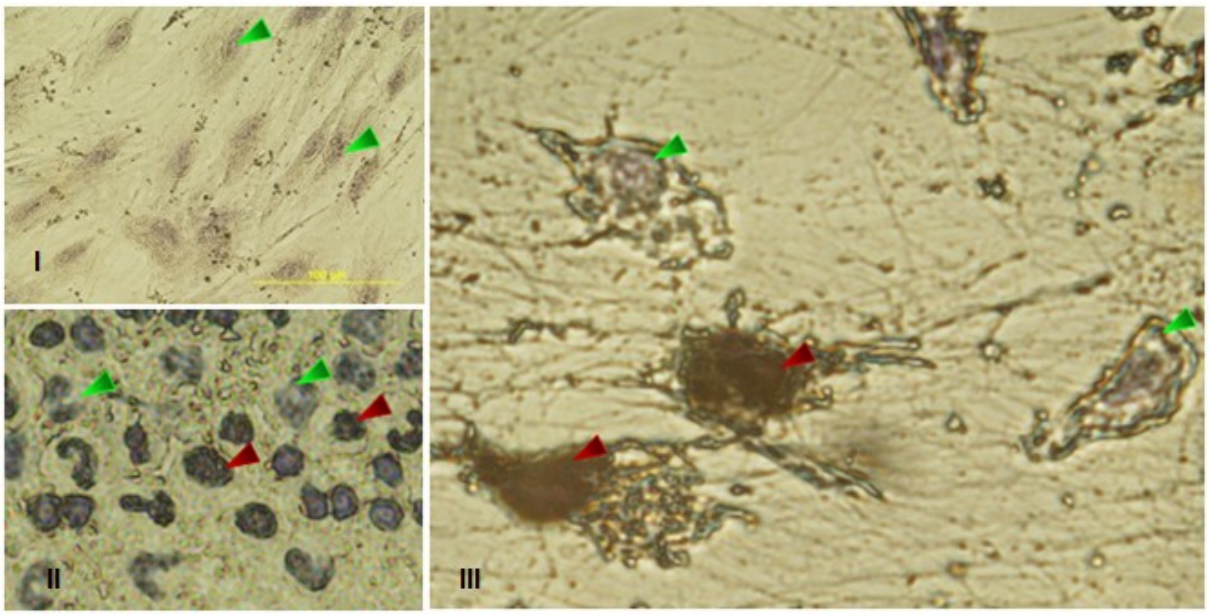


Fig. 6 HD cells exhibit the activity of the macrophage specific enzyme α-NSE, which is detectable by characteristic brown cytoplasmatic stain (red arrows). I: Undifferentiated ASCs are exclusively H&E positive and negative for α-NSE activity (green arrows). II: Contrarily, the slide prepared from BM cells contains α-NSE positive (red arrows) and negative cells (green arrows). III: After 12 days of hematopoietic differentiation, 36.27± 4.36% of the HDs display a distinct activity of α-NSE, verified by a positive staining result (red arrows).

Macrophages, as effector cells, play a key role in the inflammatory response and release a broad number of cytokines in response to major stimulators, such as IL-4 and lipopolysaccharide (LPS). To elucidate if HD cells exhibit such phenomenon, these cells were stimulated with either 20ng/ml IL-4 for 5 days or with 1 µg/ml LPS derived from *E. Coli* (strain O127:B8) for 3 days. IL-4 stimulation altered the morphology and size of the cells (Fig. 7a). The percentage of cells sized >30 micron diameter increased from 2% to about 14% upon 75 hours of stimulation (Fig. 7b and c). Analysing the effect of IL-4 on the genetic profile of differentiated cells revealed a strong induction of HLA DRB1 gene expression in HD cells. However, IL-4 attenuated the expression of macrophage specific markers MCP1 and CD68 and marginally decreased CD8 expression (about 20%); IL-4 had minimal effect on the CD4 expression (Fig. 8a and b). Similarly, LPS stimulation increased the expression of HLA DRB1. In addition, CD14 expression was intensified upon LPS stimulation, whereas CD4 expression drastically declined (Fig. 8c).

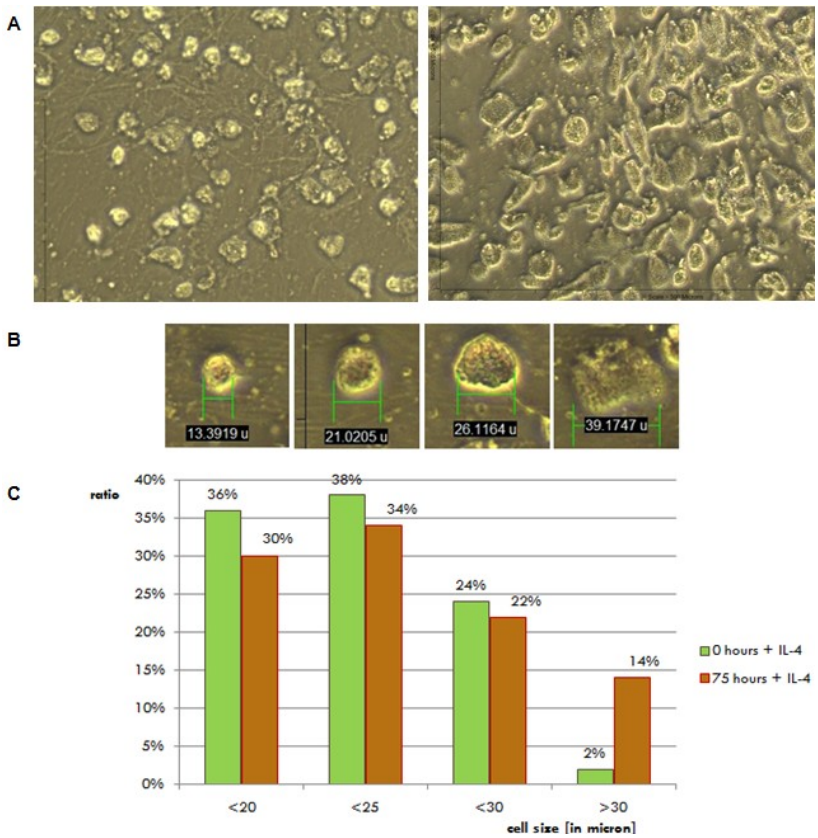


Fig. 7 HD cells respond to IL-4. (a) HD cells were treated with 20 ng/ml IL-4 for 75 h. (b) Upon stimulation, an increase in the size of HD cells was observed. (c) A measurement of the cell size substantiated this observation and revealed a decrease in the number of cells below 30µm in favor of the percentage of cells larger than 30µm, which increased significantly from 2% before stimulation to 14% after exposure to IL-4. Data represents mean values of triplicate experiments ($P < 0.05$; n=3).

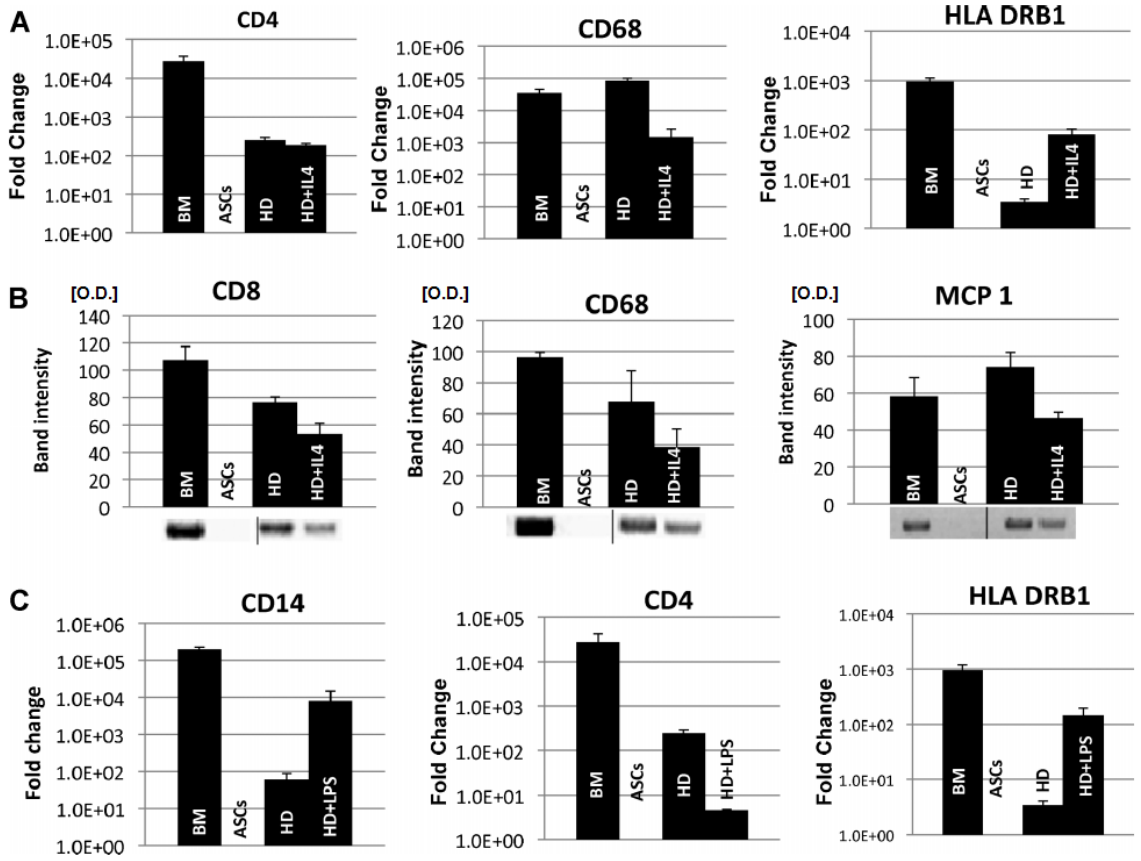


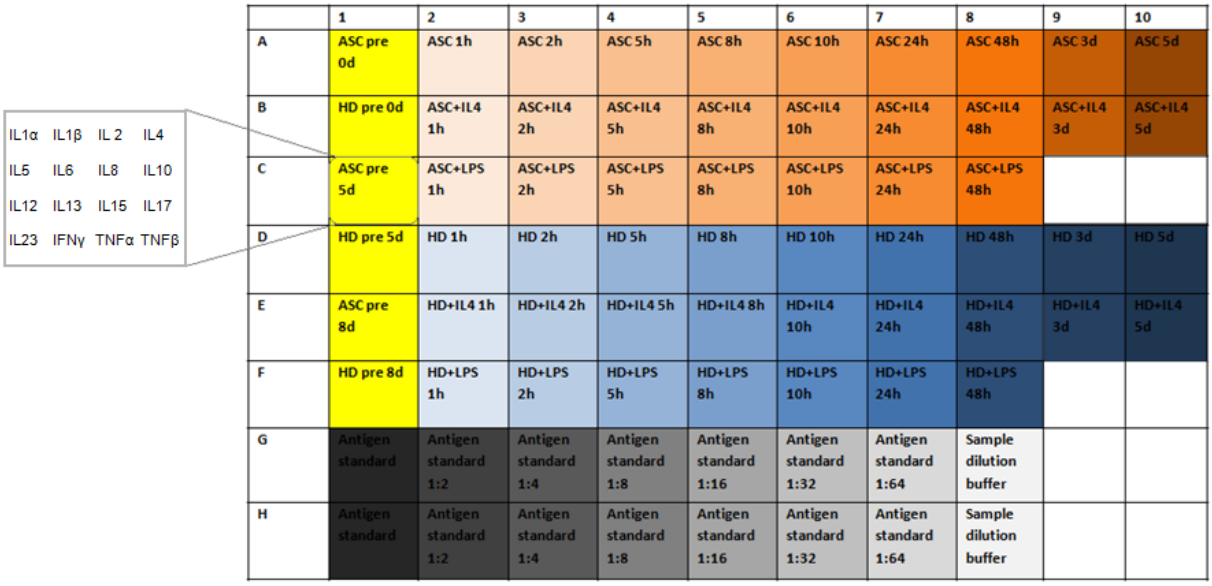
Fig. 8 Quantitative and real time analyses of gene expression in IL-4 and LPS stimulated HD cells. (a) A real- time PCR based analysis of changes in the gene expression before and after 5 days of treatment with IL-4 shows a characteristic up-regulation of HLA-DRB1 and a decrease in CD4, CD68 gene expression.(b) RT-PCR analysis show the down regulation of CD8, CD68, and MCP-1 expression in HD cells following IL-4 stimulation, the band intensities were normalized by GAPDH expression (n=6/gene; $P < 0.05$). (c) LPS stimulation induces an increased expression of CD14 and decreased CD4 expression. n=3 for independent stimulation experiments ±SD ($P < 0.05$).

A furthermore performed ELISA analysis on supernatants of both, IL-4/ LPS stimulated and non-stimulated HD cells demonstrates the time elapsed cytokine/ chemokine secretion of the differentiated cells. The cytokine responses were evaluated at multiple time points before stimulation and between 1 to 120 hours post-stimulation (Fig. 9).

HD cells appeared to exhibit an early response to LPS stimulation by increasing the secretion rates of IL-2 (~1,400pg/ml), IL-10 (~140pg/ml), and IL-17 (~300pg/ml) with a peak release at 1-2 hours post-stimulation (Fig.10a). Late response (24 hours and above) to LPS stimulation was observed by increased secretion of IL-1α (~200pg/ml) and IL-13 (~3,000pg/ml). However, LPS stimulation resulted in no significant change of IL-5, TNFα, TNFβ, and IFNγ release (Fig.10b, c). Unlike LPS, IL-4 stimulation

caused an early secretion peak of IL-13 (2-5 hours) reaching concentrations of ~1,500 pg/ml and a late response peak of IL-5 around 72 hours post-stimulation (up to ~250 pg/ml). IL-1α secretion seems to be suppressed by IL-4 (~250pg/ml) which remained below the secretion level in un-stimulated HD cells. However, there was a small increase in TNFα and IFNγ levels around 8 hours post exposure to IL-4. The level of IL-17 secretion was not affected in HD cells by IL-4.

Furthermore, non-stimulated HD cells released significant amounts of IL-2 (up to ~750 pg/ml) and IL-10 (~150pg/ml) which rapidly declined after ~8 days (Fig. 10d). Upon stimulation with LPS IL-2, IL-10, IL-17 and TNFβ were detectable in undifferentiated ASCs. However, the expressions of these cytokines were significantly lower than in HD cells. Stimulation of undifferentiated ASCs by IL-4 resulted in increased secretions of IL-13, IL-17, and TNFα and decreased IL-2 expression. There was no detectable change in IL-1α secretion upon IL-4 or LPS stimulation in undifferentiated ASCs (Fig.10e). IL-6, IL-8, IL-12p70, IL-15 and IL-23 release were not detectable (data not shown).



	1	2	3	4	5	6	7	8	9	10
A	ASC pre 0d	ASC 1h	ASC 2h	ASC 5h	ASC 8h	ASC 10h	ASC 24h	ASC 48h	ASC 3d	ASC 5d
B	HD pre 0d	ASC+IL4 1h	ASC+IL4 2h	ASC+IL4 5h	ASC+IL4 8h	ASC+IL4 10h	ASC+IL4 24h	ASC+IL4 48h	ASC+IL4 3d	ASC+IL4 5d
C	ASC pre 5d	ASC+LPS 1h	ASC+LPS 2h	ASC+LPS 5h	ASC+LPS 8h	ASC+LPS 10h	ASC+LPS 24h	ASC+LPS 48h		
D	HD pre 5d	HD 1h	HD 2h	HD 5h	HD 8h	HD 10h	HD 24h	HD 48h	HD 3d	HD 5d
E	ASC pre 8d	HD+IL4 1h	HD+IL4 2h	HD+IL4 5h	HD+IL4 8h	HD+IL4 10h	HD+IL4 24h	HD+IL4 48h	HD+IL4 3d	HD+IL4 5d
F	HD pre 8d	HD+LPS 1h	HD+LPS 2h	HD+LPS 5h	HD+LPS 8h	HD+LPS 10h	HD+LPS 24h	HD+LPS 48h		
G	Antigen standard	Antigen standard 1:2	Antigen standard 1:4	Antigen standard 1:8	Antigen standard 1:16	Antigen standard 1:32	Antigen standard 1:64	Sample dilution buffer		
H	Antigen standard	Antigen standard 1:2	Antigen standard 1:4	Antigen standard 1:8	Antigen standard 1:16	Antigen standard 1:32	Antigen standard 1:64	Sample dilution buffer		

Fig. 9 Experimental setup of ELISA based cytokine release. Supernatants of HD cells (blue) and ASCs (red) were collected after 1h, 2h, 5h, 8h, 10h, 48h of stimulation with LPS and additionally after 3 and 5 days post IL-4 stimulation. To each time point, the matching supernatants of non stimulated cultures (ASCs and HDs) served as a control. For baseline levels, supernatants of unstimulated ASCs and HD cells of different maturation levels (0d, 5d, 8d in culture) were included (yellow). Each well detected the cytokines IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, Il-10, IL-12, IL-13, IL-15, IL-17, IL-23, IFNγ, TNFα, TNFβ (box). For quantitative analysis, a dilution series of antigen standard containing accurately defined contents of these cytokines was prepared in duplicate (grey).

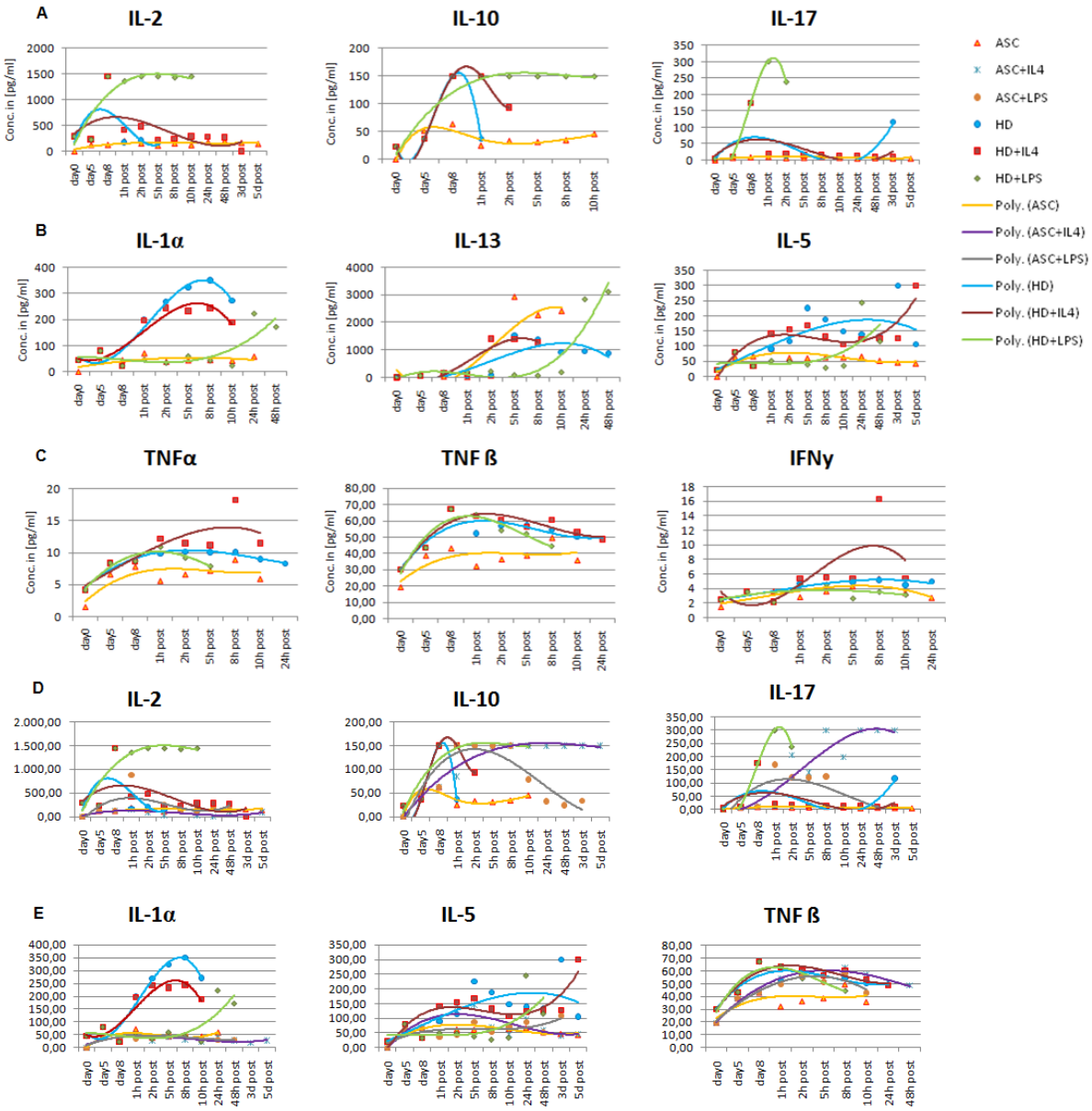


Fig. 10 Cytokine release from HD cells in response to IL-4 and LPS stimulation. Culture media was harvested on days 0, 5, and 8 of differentiation. In addition, subsequent to treatment of cultures with either IL-4 (20 ng/ml) or LPS (1mg/ml), supernatants were harvested at 1, 2, 5, 8,10, 24, 48, 72, and 120 h post stimulation. Supernatants were then assayed for cytokine content using ELISA. (a) LPS stimulation caused an early increase in the release of IL-2, IL-10, and IL-17. (b) Exposure to IL-4 predominantly induces an augmented secretion of IL-5 and IL-13 and a decrease in concentration of IL-1α. TNFα, TNFβ, and IFNγ showed low secretion rates and minimal response to both stimuli (b, c). Non-stimulated HD cells released significant amounts of IL-2 and IL-10 (d), there was no detectable change in IL-1α secretion upon IL-4 or LPS stimulation in undifferentiated ASCs (e).

x-Axis shows the concentration of secreted cytokines (pg/ml), y-axis represents time in which the concentrations were analyzed. Data were analyzed using Q-View Plus software from Quansys Biosciences and depicted by polynomial regression graphs (order 3).

Prospect: HD cells are permissive to HIV-1 infection

Macrophages, as components of the innate cellular immune system, are involved in host response to infection with pathogens. They are known to be one of the most important targets for HIV-1 infection, the etiologic agent of acquired immune deficiency syndrome (AIDS) and play a crucial role in viral infectivity, latency, pathogenesis and recrudescence. HIV infection of lymphoid and myeloid hematopoietic lineages is mediated by recognition of the T-cell receptor CD4 or by the chemokine co-receptors CXCR4 and CCR5. Whereas CCR5 appears to be the most important receptor for HIV-1 entry into monocytes and macrophages (M-tropic), it is CXCR4 for T-lymphocytes (T-tropic) [40].

From transcriptome analysis, it is evident that HD cells express a number of genes that encode HIV-1 target proteins and key cellular cofactors (e.g. CD4, CCR5, CD32, CD64) [40,41]. Thus, the susceptibility of HD cells and undifferentiated ASCs (as their cellular origins) towards HIV-1 infection were examined, as well as their successive capability to support viral replication.

Results: HIV-1 infected HD cells (HD-HIV) showed elevated p24 protein and Tat gene expression (Fig. 11), implying a high productive infection. Undifferentiated ASCs were not susceptible to HIV-1. Further, HIV-infection altered the gene expression in HD cells and increased apoptosis.

This work was expanded by our research group in a subsequent study. The entire study has recently been published [42].

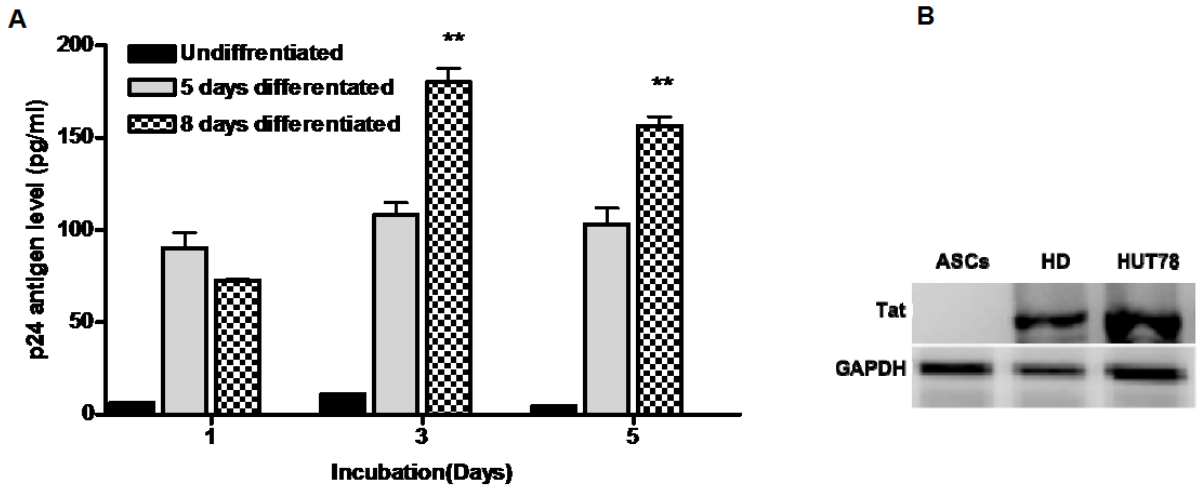


Fig. 11 Infection of HD cells with HIV-1. (a) HIV-1 was exposed to undifferentiated, 5 day and 8 day hematopoietic differentiated ASCs for 24h, when p 24 antigen level was detected after 1 , 3 and 5 days after removal of the virus. Data represents the compilation of two separate experiments done in duplicates. (b) Unlike ASCs, HD cells showed Tat gene expression after HIV-1 exposure. HUT78 cells served as positive control (Nazari-Shafti, Freisinger et al., 2011)

Discussion

In this study, it could be shown that culturing ASCs in a newly developed differentiation media supplemented with hematopoietic promotors results in generation of cells which exhibit morphological and functionally distinct hematopoietic characteristics.

Several studies have suggested that phenotypically normal and functionally competent cells of the hematopoietic system could be derived from embryonic stem cells (ESCs), bone marrow HSCs, or peripheral blood HSCs [20,31]. It has previously been reported that adipose tissue derived stem cells express angiogenic and hematopoietic factors [23]. Additionally, there have been a number of reports indicating the presence of a non-adherent subset of cells within adipose tissue that display hematopoietic activity [25,26,43-47]. Therefore, multipotent adipose tissue derived mesenchymal stem cells appear to be an eligible candidate for trans- differentiation towards the hematopoietic lineage, and more precisely towards macrophages.

The primary ASC population was isolated from a cohesive piece of abdominal adipose tissue in order to reduce the potential for contamination with hematopoietic cells, which, given the traumatic nature of the harvest, will typically be found within lipoaspirate samples. It is known that this contamination gets eliminated in adherent MSC cultures within the three initial passages under maintenance media culturing conditions [9,13]. To exclude any possibility of hematopoietic contamination, the clones were examined for hematopoietic specific surface markers (CD34, CD45, CD68, CD11b) during the course of study and for 5 consequent passages. In addition, adipogenic, osteogenic, hepatogenic, chondrogenic and neurogenic differentiation assays confirmed the multilineage character of the clones and therefore their classification as ASCs (IFATS criteria).

In the undifferentiated state, the ASC clones used in this study expressed positive mesenchymal markers and none of those associated with a hematopoietic phenotype ($CD44^+$, $CD90^+$, $CD105^+$, $CD45^-$, $CD34^-$, $CD4^-$, $CD11b^-$, $CD14^-$ and $CD68^-$).

First, a suitable culture media for hematopoietic differentiation had to be developed. Based on published protocols, a comparative analysis of the used components revealed the importance of GM-CSF, M-CSF, IL-3 to induce myeloid characteristics on cells. IL-1β as an intense pro- inflammatory cytokine is very likely to support further maturation and activation [37]. In addition, 1-MTG has been used as reducing agent in order to promote differentiation [35]. In order to provide optimal growth conditions, the differentiation media is based on α-MEM, supplemented with FBS comparable to maintenance media for (undifferentiated) ASCs.
The exact concentrations of the components have been investigated by multiple trials under various configurations. Hereunder, the cultures were evaluated for confluency, cell growth, morphology and adherence by microscopy and additionally for cell viability analysis using Trypan Blue staining. On the basis of these criteria the optimal composition of the differentiation media could be identified.
Stable differentiation combined with high cell viability could be found upon 100 U/ml IL-1β, 500 U/ml IL-3 and 20 U/ml M-CSF supplemented with 0.1 µl/ml 1-MTG.

Culturing ASC clones in a mixture of cytokines resulted in a significant alteration in morphology from spindle to round shaped cells. ASCs are adherent growing under regular culturing conditions. Under hematopoietic differentiation conditions, cells gradually lost their adherence capabilities. This phenomenon has previously been reported on human peripheral blood monocytes upon stimulation with IL-3 and M-CSF [48,49]. The components of the differentiation media, such as IL-1β, IL-3, M-CSF, and MTG, play a crucial role in macrophage development [49-52] and therefore have previously been used to induce macrophage differentiation in HSCs and ESCs *in vitro* [20,33] and *in vivo* [53].

Genetics

The transcriptomic analysis of HD cells indicated a genomic shift towards the hematopoietic lineage as shown by the expression of c-KIT and PROM-1. These genes together with CD4 are known to be associated with hematopoiesis of HSCs in bone marrow [54]. GATA and PU.1 mRNAs were only weakly expressed in HD cells. It has been reported that these two genes display myeloid and granulocyte/ monocyte/ lymphoid potential in HSCs [55]. Upon differentiation, HD cells have developed receptors for IL-1β and IL-3. IL-1β receptor binds to IL-1β, which is a

primary regulator of inflammation and inflammatory responses. Furthermore, it has been shown that IL-1β receptor plays an important role in the regulation of transcription factors such as nuclear factor kappa-B (NFkB) [56]. Binding IL-3 to its receptors initiates the formation of a high-affinity receptor complex, binding the respective ligand in the range of its physiologic concentrations and to transduce proliferative, anti-apoptotic and differentiative signals [57,58].

Additionally, the pronounced expression of the chemokine receptor CCR5, IFNGR1, CD14, MCP1, HLA- DR B1 and to lower extent CD32 and CD64 evinces the tendency of HD cells to differentiate into immunocompetent cells such as granulocytes, T-cells or monocytes/ macrophages. Moreover, HD cells possess the genomic and proteomic profile to percept pathogen-associated structures, such as LPS (via CD14) and to opsonize IgG Fc fragments (via CD32 and CD64). The expression of HLA-DR refers to the antigen presenting cell (APC) characteristic of macrophages. Most significantly, the expression of CD68 and CD11b as specific monocyte/ macrophage markers, substantiated by MRC1, CSF1R, IL-10, and TNF up-regulations, characterizes the HD cells as being similar to macrophages.

Proteomics

Immunocytochemistry data confirmed the presence of CD68, CSF1R and the macrophage specific NOS2 protein. Csf1R not only mediates the various effects of the cytokine CSF-1 but also has been shown to selectively promote macrophage maturation during differentiation of HSCs [59]. NOS2 encodes the nitric oxide (NO) synthase which is inducible by LPS. It is known that some cytokines induce NO production as part of the oxidative burst in macrophages.

Functionality

Moreover, the enzymatic activity of α-NSE, a member of cellular carboxyl esterases, and an enzymatic marker of monocytes/macrophages [60], could be very well detected in the HD cells. Although the exact role of α-NSE has not been completely described yet, it is known to be associated with inflammatory processes and may play a role in the pathogenesis of chronic inflammatory diseases [61].

The detected expression of CD68 and CD11b, together with CD14 and MRC1, suggests the ability of HD cells to ingest particles, since these genes are heavily involved in mediating the process of phagocytosis in hematopoietic derived macrophages. A phagocytosis assay with fluorescent-labeled *E. coli* particles displayed an uptake of particles in > 95% of HD cells. Thus, hematopoietic differentiation results in the development of phagocytosis as one of the most important key functions of macrophages.

In HD cells, the detection of TNFα, CCR5, IL10 and CD14 gene expression indicates a cellular response upon pro- and anti-inflammatory stimuli, which has been investigated in the following by rt RT-PCR and a functional ELISA based assay.

LPS, as a component of the bacterial membrane, is known to be a potent activator of immunocytes, effective via CD14, TLR-2, TLR-4, and LY-86 (MD-2) receptors [62]. LPS initiates the classical activation of macrophages and leads to the release of pro-inflammatory cytokines, such as IL-1, IL-6, TNFα but also MCP1 and IL-10 [49,63,64]. HD cells were stimulated with 1 µg/ml of LPS. It has been shown that newly differentiated macrophages from ESCs are responsive to this concentration of LPS [20]. Similarly, as seen in macrophages, HD cells respond to LPS exposure by a strong up-regulation of its receptor CD14 [65]. The expression of CD4 in LPS stimulated HD cells decreased simultaneously, as it has previously been reported in primary human macrophages [66]. Data obtained from stimulation experiments also indicate an increase in the concentration of IL-2, IL-10, and IL-17 in LPS stimulated HD cells.

IL-4 activates the alternative pathway, which is associated with a predominantly anti-inflammatory microenvironment (e.g. release of IL-5, IL-13 but a down- regulation of IL-1, IL-6, IL-8 and TNFα) [20,62,67-70]. IL-4 stimulation of HD cells resulted in a characteristic up-regulation of HLA DRB1 expression [71,72]. The increase of major histocompatibility complex class II in stimulated HD cells, an important factor in presenting extracellular antigens, is similar to the capacity of macrophages functioning as APCs. Moreover, it is known that IL-4 actively promotes the differentiation of monocytes to dendritic cells as the most potent APCs [73]. In this context an increasing cell size has been described upon stimulation with IL-4 which corroborates the same phenomenon we observed in IL-4 stimulated HD cells [49,71,74].

IL-4 stimulation further diminished CD68, CD4 and CD8, and MCP-1 expression

which is congruent with findings on human monocytes [75,76]. Moreover, the enhanced release of IL-5 and IL-13 and the decreasing concentration of IL-2 upon IL-4 stimulation support this data.

It has been shown that undifferentiated ASCs can also be stimulated with LPS and IL-4 [23], however, our data show the ASC response to be considerably lower and/or can be found at a later time point. Interestingly, following IL-4 stimulation, IL-13 and the low secreted cytokines (TNFα, TNFβ, IFNγ) were detected in comparatively higher concentrations in undifferentiated ASCs. Since macrophages themselves are extremely heterogeneous and react miscellaneously on certain stimuli or microenvironments [77-80], determination of the actual maturity level of HD cells is difficult. However, our data show that the HD cells are responsive to stimuli via both classical and alternative pathways.

As showed in an adjacent study, HD cells are - unlike ASCs - permissive to HIV-1 infection.

In summary, it could be verified that it is possible to generate cells under specific culturing conditions that exhibit distinct genetic, proteomic and functional characteristics of monocytes/ macrophages.

Conclusion

Data presented in this study provide ample evidence that a single cell of multipotent mesenchymal stem cells derived from human adipose tissue is capable to differentiate into a cell of hematopoietic lineage *in vitro*.

These from ASCs generated hematopoietic cells could serve as a valuable *in vitro* model to further understand the mechanisms involved in cell-cell interactions within the immune system, especially under the phathological conditions of infectious diseases and immune disorders. Further, macrophages were identified to be involved in the development of atherosclerotic lesions [81], thus HD cells could serve as a cellular model for atherogenesis and for pharmaceutical research.

Future Prospects

Eventually, hematopoietic differentiated ASCs could be used as autologous cell transplants in the future, especially regarding hematologic disorders (e.g. leukemia, osteomyelofibrosis), here more precisely in macrophage cell disorders such as Whipple's Disease [82] or Erdheim-Chester Disease [83]. Regarding their vulnerability for HIV-1 infection, HD cells could be used as possible tool for pharmaceutical research and probably for future gene therapy against HIV-1. Lately, the generation of HIV-1 resistant and functional macrophages has been reported for pluripotent hematopoietic stem cells *in vitro* [84]. Viral suppression upon allogenic HIV-resistant HSC transplantation in a case report [85,86] substantiates the prospective significance of stem cells as a possible therapeutic option.

In general, the lower probability for an immune rejection by using autografts and lesser reason for ethical conflicts compared to the use of embryonic stem cells or homologous transplants are just some of the many benefits in the use of ASCs. Adipose tissue derived mesenchymal stem cells can easily be obtained in large quantities with minimal risk and discomfort for the patient and show a high replication rate in culture making them versatile applicable.

After all, this study contributes to the current understanding of the development and characteristics of adult stem cells. The novel finding that human ASCs can be induced to express hematopoietic genes modifies the current idea of the bone marrow as a unique organ to generate hematopoietic cells. Regarding the presented data it can be hypothesized that the endocrine active adipose tissue functions as a reservoir for adult MSCs, thus to substitute mesodermal cells including hematopoietic cells as exposed to an adequate microenvironment.

Schlussfolgerungen

Die im Rahmen der vorliegenden Studie erhobenen Daten belegen die Differenzierung hämatopoetischer Zellen *in vitro* aus adipogenen multipotenten mesenchymalen Stammzellen des Menschen.

Diese aus ASCs generierten hämatopoetischen Zellen könnten als wertvolles *in vitro* Modell dienen, um weitere Erkenntnis über die Mechanismen der Zell-Zell-Interaktionen innerhalb des Immunsystems zu erlangen, insbesondere unter den pathologischen Bedingungen von Infektionen oder immunologischen Erkrankungen.
Weiterhin wird Makrophagen eine tragende Rolle bei der Entwicklung atherosklerotischer Plaques zugeschrieben [81], wonach HD-Zellen als zelluläres Modell für Atherogenese wie auch für pharamzeutische Studien auf diesem Gebiet Verwendung finden könnten.

Ausblick

Möglicherweise könnten zukünftig autologe Zelltransplantation hämatopoetisch differenzierter ASCs zur Therapie hämatologischer Erkrankungen (z.B. Leukämie, Osteomyelofibrose) und insbesondere von Erkrankungen, welche das Monozyten/Makrophagen-System betreffen wie Whipple's Disease [82] oder Erdheim-Chester Disease [83] eingesetzt werden. Hinsichtlich der Vulnerabilität von HD-Zellen gegenüber einer HIV-1 Infektion wäre ein Nutzen sowohl als Zellmodell zur pharmakologischen Forschung als auch möglicherweise zukünftig im Rahmen angewandter Stammzelltherapie bei HIV/ AIDS denkbar. Kürzlich wurde von der Generierung HIV-1 resistenter und funktionaler Makrophagen aus pluripotenten hämatopoetischen Stammzellen *in vitro* berichtet [84]. Eine virale Suppression durch allogene Transplantation HIV-resistenter HSCs in einem Fallbericht[85,86] untersützt die Aussicht auf ein künftig hohen Stellenwert der Stammzellen als mögliche therapeutische Option.

Insgesamt stellen die geringere Wahrscheinlichkeit einer immunologischen Abstoßungsreaktion bei der Verwendung von Autografts als auch der geringere

damit einhergehende ethische Konflikt gegenüber der Verwendung embryonaler Stammzellen oder homologen Transplantate nur einige der vielen Vorteile der ASCs dar. Adipogene mesenychmale Stammzellen können auf einfachem Weg in großer Zahl gewonnen werden, und dies unter minimalem Risiko und Diskomfort für den Patienten. Zudem zeigen ASCs hohe Replikationsraten in Kultur, was sie vielseitig einsetzbar macht.

Zusammenfassend betrachtet kann im Rahmen der vorliegenden Studie nachgewiesen werden, dass es möglich ist, unter speziell dafür entwickelten Kultivierungsbedingungen Zellen zu generieren, welche sowohl genetisch, auf Proteinebene als auch hinsichtlich ihrer Funktionalität spezifische Charakteristika von Monozyten/ Makrophagen aufweisen.

Die vorgelegten Ergebnisse tragen zum Verständnis von Entwicklung und Charakterisierung adulter Stammzellen bei.

Die neue Erkenntnis, dass humane ASCs induziert werden können, hämatopoetische Gene zu exprimieren, relativiert die aktuell vorherrschende Sichtweise des Knochenmarks als einziges Organ, welches zur Hämatopoese befähigt ist. Angesichts der ermittelten Daten kann die Hypothese aufgestellt werden, dass ein endokrin aktives Fettgewebe als Reservoir für adulte MSCs und somit der Substitution mesodermaler Zellen, inklusive hämatipoietischer Zellen dienen kann, sofern eine Exposition gegenüber einem geeigneten „Mikroenvironment“ besteht.

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Declaration

Ich erkläre, dass ich die der Medizinischen Fakultät der Otto-von-Guericke-Universität zur Promotion eingereichte Dissertation mit dem Titel

„ *Generation of Functional Macrophages from Adult Mesenchymal Stem Cells* “

am Applied Stem Cell Laboratory, Heart and Vascular Institute, Department of Medicine, Tulane University Health Science Center New Orleans, Louisiana, U.S.A.

ohne sonstige Hilfe durchgeführt und bei der Abfassung der Dissertation keine anderen als die dort aufgeführten Hilfsmittel benutzt habe.

Bei der Abfassung der Dissertation sind Rechte Dritter nicht verletzt worden.

Ich habe diese Dissertation bisher an keiner in- oder ausländischen Hochschule zur Promotion eingereicht. Ich übertrage der Medizinischen Fakultät das Recht, weitere Kopien meiner Dissertation herzustellen und zu vertreiben.

Magdeburg, den

Unterschrift

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Journal of Cell Physiology

"Characterization of Hematopoietic Potentials of Mesenchymal Stem Cells" (Freisinger E, Cramer C, Xia X, Murthy SN, Slakey DP, Chiu E, Newsome ER, Alt E, Izadpanah R)

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"Stem Cells and Aging: Physical and Genetic Manifestations" (Co-Author)