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Research Article

Comprehensive Screening of Cell Surface Markers Expressed by Adult-Derived Human Liver Stem/Progenitor Cells Harvested at Passage 5: Potential Implications for Engraftment

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Mesenchymal stromal cells (MSCs) are known to have potential therapeutic benefits for a number of diseases. However, many studies report low engraftment levels, regardless of the target organ. One possible explanation could be that MSCs do not express the necessary receptors for engraftment. Indeed, MSCs appear to use a similar mechanism to leukocytes to engraft into injured organs, relying on various receptors for rolling, firm adhesion, and transmigration. In this study, we conducted an extensive surface molecule screening of adult-derived human liver stem/progenitor cells (ADHLSC) in an attempt to shed some light on this subject. We observed that ADHLSCs lack expression of most of the costimulatory molecules tested. Furthermore, study of the adhesion molecule profile of ADHLSCs revealed that they do not express selectin ligands or LFA-1 which are, respectively, involved in the rolling process and the firm adhesion. In addition, ADHLSCs slightly express VLA-4 and lose expression of CXCR4 altogether on their surface during culture expansion. However, ADHLSCs express all the integrin couples and matrix metalloproteinases needed to bind and integrate the extracellular matrix once the endothelial barrier is crossed. Collectively, these results suggest that binding to the endothelium may be the critical weak point in the engraftment process.

1. Introduction

Mesenchymal stromal cells (MSCs) have been isolated and characterized from various sources (liver, heart, lung, and bone marrow) [1]. Some of them are currently being investigated for cell therapy in the treatment of a wide range of diseases (cancer, heart stroke, inflammatory diseases, and genetic disorders). Our group has previously isolated and characterized stem/progenitor cells from healthy adult human liver (ADHLSCs) [2, 3]. These expandable cells show a hepatomesenchymal phenotype and have the potential to differentiate into hepatocyte-like cells both in vitro and in vivo [2, 4, 5]. ADHLSCs are now in phase 2/3 of clinical trials to treat inborn errors of metabolism of the liver such as urea cycle disorders or Crigler Najjar syndrome.

However, as is the case with most mesenchymal stem/progenitor cell-based therapies, the rate of engraftment of ADHLSCs into the recipient liver remains low [4]. One hypothesis is that donor cells could be cleared by the immune system of the recipient. Our previous studies indicated that ADHLSCs are poorly immunogenic [6, 7], but their immune profile has not yet been completely characterized. In addition, there could be some impairment in the engraftment process itself. A number of studies suggest that the engraftment process of MSCs is similar to that of leukocytes or hematopoietic stem cells (HSCs). The cells pass through a rolling phase, followed by a firm adhesion step, and finally transmigration through the endothelium [8, 9], which takes 10 to 20 minutes for leukocytes and 60 to 120 minutes for MSCs [10]. Unlike leukocytes, MSCs do not express the same number of

adhesion molecules to accomplish this engraftment process. First, MSCs do not express the selectin ligands required to slow them down on activated endothelium [11]. Second, they do not express lymphocyte function-associated antigen 1 (LFA-1), which would allow them to bind to intercellular adhesion molecule 1 (ICAM-1) on endothelial cells. However, during inflammation, the activated endothelium secretes stromal cell-derived factor 1 (SDF-1), which increases recruitment of MSCs through C-X-C chemokine receptor type 4 (CXCR4) and can also activate cells and help in the firm adhesion step mediated by very late antigen-4/vascular cell adhesion protein 1 (VLA-4/VCAM) [12, 13]. Transmigration through the endothelium appears to rely on VLA-4/VCAM binding, followed by use of matrix metalloproteinases to integrate into the organ. We have previously shown that ADHLSCs express some adhesion molecules [2, 7], but information was still lacking on a number of key receptors involved in the engraftment process. In addition, the requirements of large scale cultures for clinical use have prompted us to move from culture on collagen-coated flasks and an emergence in the presence of EGF, to culture on CellBIND® plastic, treated to facilitate adhesion.

In the current study, we performed extensive screening of all ADHLSC surface antigens using the BD Lyoplate™ human cell surface marker screening panel following culture in large scale conditions. This screening also allowed us to complete their surface marker characterization, confirm their low expression of immunogenic markers, and shed light on potential weak points in the ADHLSC engraftment process.

2. Materials and Methods

2.1. ADHLSC Isolation and Culture. The protocol and experiments were approved by the ethics committees of the St Luc's University Hospital and the Faculty of Medicine of the Université Catholique de Louvain. Approval from the Belgian Ministry of Health was obtained for the hepatocytes and hepatic stem cells bank. Written and signed informed consent was also obtained for each human liver used in the current study.

Eight donors were used in the current study (Table 1). ADHLSCs were recovered subsequent to primary culture of the liver parenchymal fraction achieved after two-step collagenase perfusion, filtration, and low-speed centrifugation, as described elsewhere [2]. ADHLSCs were cultured on CellBIND flasks (Corning®) in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/L glucose (Invitrogen), supplemented with 10% fetal calf serum (Gibco) and 1% penicillin/streptomycin (Invitrogen), at 37°C in a fully humidified atmosphere (5% CO_2). Upon reaching 80% confluence, cells were lifted with 0.05% trypsin-EDTA (Invitrogen) and replated at a density of 5000 cells/cm^2. The viability of the recovered cells was evaluated using the trypan blue dye exclusion method.

2.2. Cell Surface Marker Screening by Flow Cytometry Using BD Lyoplate Technology. The BD Lyoplate human cell surface marker screening panel (BD Biosciences, Heidelberg, Germany) was used to characterize cultured ADHLSCs. The kit contains 242 purified monoclonal antibodies to cell surface markers, as well as isotype controls to assess nonspecific backgrounds. Before use, plates containing lyophilized antibodies were centrifuged at 300 ×g for 5 minutes. The antibodies were then reconstituted in 110 μL of sterile Dulbecco's Phosphate-Buffered Saline (DPBS).

Table 1: Characteristics of the 8 liver donors from which ADHLSCs were isolated.

Donor number	Age	Gender	Reason of death	Blood group
15	25 years	M	/	A+
89	3 days	M	Respiratory	A+
93	2 years	F	Metabolic disease	O+
98	7 days	M	Cardiorespiratory arrest	O−
105	46 years	F	Traumatism	B+
107	4 days	F	Nonketotic hyperglycemia	O+
115	3 months	M	Meningitis	O+
116	6 days	F	Neonatal asphyxia	O+

The assay was performed on five donors, according to the manufacturer's instructions. Briefly, ADHLSCs were harvested at passage 5 using 0.05% trypsin-EDTA. After washing in DPBS, cells were resuspended in Pharmingen stain buffer containing 5 mM EDTA at a concentration of 1.25×10^6 cells/mL. Eighty microliters of cell suspension per well was then transferred to 96-well plates and stained with 20 μL of specific primary antibodies for 30 minutes on ice. Thereafter, the cells were washed twice with Pharmingen stain buffer + 5 mM ETDA and stained with 100 μL of Alexa Fluor 647-labeled anti-mouse or anti-rat secondary antibody (diluted 1 : 200 in Pharmingen stain buffer + 5 mM EDTA) for 30 minutes on ice. After washing, the cells were fixed with BD cytofix fixation buffer and transferred from the 96-well plates to single BD FACS tubes. Fluorescence was measured with a BD FACSCanto II cytometer on 10,000 cells using FACSDiva software.

For analysis, background fluorescence was set manually for each sample based on its appropriate isotype using FlowJo software. Results are expressed as a percentage of positive cells in the population or median fluorescence intensity (MFI).

2.3. Analysis of CD184 and CD90 Expression by Flow Cytometry. For cell surface staining, liver cells were first incubated with DPBS-bovine serum albumin (BSA) 1.5% for 20 minutes at 4°C to prevent nonspecific binding. Next, the cells were washed with DPBS-BSA 1.5% and stained with 5 μL of PE rat anti-human CD184, APC mouse anti-human CD90, or their respective isotypes (BD Biosciences) for 30 minutes on ice. Finally, the cells were washed and fixed using a stabilizing fixative (BD Biosciences). For intracellular staining, liver cells were fixed and permeabilized with 200 μL

of cytofix/cytoperm buffer (BD Biosciences) for 20 minutes at 4°C. The cells were then washed with perm/wash buffer and stained with PE rat anti-human CD184 or its isotype diluted in perm/wash for 30 minutes on ice. Next, the cells were washed twice and fixed with stabilizing fixative (BD Biosciences). Fluorescence was measured with a BD FACSCanto II cytometer on 10,000 cells using the FACS-Diva software. Data analyses were performed with FlowJo software.

2.4. Immunofluorescence. ADHLSCs were plated at passage 4 on 8-chamber slides (BD Biosciences) at a density of 5,000 cell/cm^2. Upon reaching 70% confluence, they were fixed with 4% paraformaldehyde for 15 minutes. After 2 washes with DPBS, ADHLSCs were blocked with DPBS-BSA 5% for 1 hour. Some of the samples were permeabilized with Triton 0.1% DPBS-BSA 1.5% buffer (BD Biosciences) for 20 minutes at 4°C. The cells were then stained with a PE rat anti-human CD184 for 2 hours at 4°C (BD Biosciences) and rinsed 3 times with DPBS. Finally, samples were embedded in ProLong Gold with DAPI (BD biosciences). Pictures were taken with an Axio Imager + ApoTome (Zeiss) at a 20x objective and analyzed with AxioVision software.

2.5. Real-Time PCR. Total RNA was extracted from 4 ADHLSC donors at passage 5 using TriPure isolation reagent (Roche, Mannheim, Germany), following the manufacturer's instructions. Briefly, 1.5×10^6 cells were homogenized in TriPure reagent, mixed with chloroform, shaken vigorously for 15 second, and centrifuged at 12,000 ×g for 15 minutes at 4°C. RNA in the upper aqueous phase was precipitated by isopropanol, washed in 75% ethanol, air-dried, and dissolved in RNase-free water. RNA samples were stored at −80°C after quantification with a NanoDrop 2000 spectrophotometer (Thermo Scientific).

cDNA was synthesized from 1 μg of total RNA by reverse transcription polymerase chain reaction (RT-PCR) using a high-capacity kit (Applied Biosystems). Thereafter, 10 ng of RT product was deposited in each well of a TaqMan® array human extracellular matrix and adhesion molecules (Invitrogen), as instructed by the manufacturer. Plates were read using the Applied Biosystems StepOnePlus real-time PCR system. The PCR data were normalized with the house-keeping gene GUSB (Glucuronidase Beta).

2.6. Statistical Analysis. Experimental data were expressed as the mean ± SEM and were analyzed using two-way analysis of variance (ANOVA). Values of $P < 0.05$ were considered to be of statistical significance.

3. Results and Discussion

3.1. Mesenchymal Phenotype of ADHLSCs. Screening performed on five donors using the BD Lyoplate confirmed that ADHLSCs harvested at P5 express CD73, CD90, and CD105 but lack CD11b, CD14, CD19, CD79β, CD45, and HLA-DR expression (Figure 1 and Table 2), which are characteristics required by the International Society for Cellular Therapy to be recognized as MSCs [14]. CD90 expression was slightly lower than the 95% required, probably due to the moderate stain index of the fluorochrome provided (AF-647) resulting in a suboptimal resolution of the peaks. These results correlate with data previously shown by our team, aside from the expression of CD105, which seems to be increased after culture on CellBIND [2]. Although it would have been interesting to evaluate how the expression of the different markers tested here evolves throughout the culture process, we have focused on P5 as it is the passage currently used in the clinic. In addition, we have used a broad range of donors to represent the diversity of donors used in the clinic. Despite the differences in age and cause of death, it has to be noted that the general characteristics regarding the presence or absence of a certain marker are fairly consistent among the group; the variability lies in the degree of expression. More studies would be needed to determine the influence of donor age and cause of death on the level of expression of the markers tested here. Interestingly, a screening performed by Baer et al. [15] on adipose-derived stromal/stem cells (ASCs) using the same assay revealed a phenotype close to what we found for ADHLSCs, with the exception of a few markers expressed by ASCs and absent from ADHLSCs and bone

Table 2: Expression of MSC markers of ADHLSCs and comparison with BM-MSC and ASC.

Markers	ADHLSC	BM-MSC [14, 15]	ASC [15]
CD73	98.3% (95.0–99.7)	≥95% (guideline)	≥95%
CD90	91.5% (86.9–95.0)	≥95% (guideline)	≥95%
CD105	96.7% (93.8–99.6)	≥95% (guideline)	≥95%
CD11b	0.7% (0.0–2.0)	≤2% (guideline)	≤2%
CD14	2.0% (0.0–3.26)	≤2% (guideline)	≤2%
CD19	0.5% (0.0–2.0)	≤2% (guideline)	≤2%
CD45	1.1% (0.0–2.8)	≤2% (guideline)	≤2%
HLA-DR	0.8% ± 0.5	≤2% (guideline)	≤2%
CD34	1.3% (0.1–2.6)	0.1% (0.0–0.1)	9.0% (5.1–30.1)
CD36	3.5% (0.0–12.0)	0.1% (0.0–0.2)	11.5% (4.8–13.4)
CD91	0.7% (0.0–1.7)	N.D.	47.6% (12.7–87.4)
CD140b	93.8% (99.4–78.6)	54.3% (45.9–87.5)	79.9% (49.2–87.5)
CD141	7.2% (4.2–12.8)	N.D.	≥95%
CD201	53.3% (0.17–81.1)	2.0% (1.8–4.8)	13.6% (9.5–25.6)

Donor N°	89	93	98	105	107
Mesenchymal stromal cell marker					
CD73	98	99	100	99	95
CD90	95	94	90	87	92
CD105	94	100	99	95	96
Pluripotent stem cell markers					
CD13	97	98	87	90	93
SSEA-3	0	3	0	1	4
SSEA-4	3	1	0	3	4
TRA-1-60	0	0	0	1	1
TRA-1-81	0	0	0	2	1
Hem pro cell	5	13	2	14	6
Adhesion markers					
CD2	0	0	0	2	1
CD11a	0	0	0	2	1
CD11b	0	0	0	2	1
CD11c	0	0	0	7	0
CD15	0	0	0	2	1
CD15s	0	0	0	0	0
CD18	0	0	0	5	0
CD24	0	0	0	2	2
CD29	99	99	99	84	97
CD31	0	0	0	2	1
CD34	0	0	0	2	1
CD41a	0	0	0	1	0
CD41b	0	0	0	2	1
CD44	36	100	94	87	97
CD47	99	98	100	91	94
CD49a	5	56	48	30	13
CD49b	93	96	84	88	82
CD49c	97	90	97	85	87
CD49d	2	12	28	30	24
CD49e	99	100	100	89	95
CD49f	0	0	0	0	0
CD50	0	0	0	2	0
CD51/61	0	20	29	8	0
CD54	74	62	62	66	48
CD56	0	0	0	2	0
CD58	43	61	88	64	51
CD61	39	46	62	21	16
CD62E	0	0	0	0	0
CD62L	0	0	0	0	0
CD62P	0	0	0	2	0
CD66	0	0	0	1	0
CD66b	0	11	13	5	9
CD66f	0	0	0	1	0
CD84	0	0	0	1	1
CD97	0	0	0	1	2
CD102	0	0	0	0	0
CD103	0	0	1	0	0
CD104	0	0	1	1	0
CD106	31	5	47	14	7
CD138	0	0	0	1	0
CD144	0	0	0	1	0
CD146	16	4	67	39	11
CD147	100	100	100	99	98
CD162	0	0	0	2	1
CD164	62	67	81	66	47
CD165	52	48	74	52	28
CD166	32	84	82	72	51
CD171	0	0	0	1	0
CD321	0	4	0	3	1
CD326	0	0	0	1	0
CDw327	0	0	0	1	0
CD328	0	0	0	1	1
CDw329	0	0	0	2	0
SSEA-1	0	0	0	1	0
CLA	0	0	0	1	4
Programmed cell death/TNF receptor superfamily					
CD95	81	95	91	97	72
CD134	0	0	0	1	0
CD137 L	0	0	0	3	0
CD153	0	0	0	1	1
CD154	0	0	0	1	0
CD181	3	7	6	13	6
CD178	0	0	0	0	0
CD255	0	1	0	2	1
CD267	0	0	0	2	0
CD268	0	0	0	0	1
CD273	0	2	30	25	4
CD274	1	4	16	10	3
CD279	0	0	0	1	0
Transport protein					
CD98	97	98	99	89	92
CD338	0	0	0	2	0

Donor N°	89	93	98	105	107
Cytokine receptors					
CD25	0	0	0	0	0
CD120a	0	2	3	4	5
CD120b	0	3	0	7	0
CD121a	0	2	1	4	7
CD121b	0	0	0	2	2
CD122	0	0	0	2	4
CD123	0	0	0	2	3
CD124	0	0	0	2	3
CD126	0	0	0	2	3
CD127	0	0	0	3	0
CD128b	0	0	0	2	1
CD130	5	10	5	5	5
CD132	0	3	2	3	1
CD135	0	0	0	3	1
CD137	0	0	0	2	1
CD210	0	2	0	0	2
CD212	0	2	0	2	1
Hormone, growth factor, other protein receptors					
CD71	33	68	81	67	34
CD87	0	0	0	1	1
CD114	0	0	0	3	0
CD116	0	0	0	4	2
CD117	0	0	0	1	0
CD118	0	0	0	1	0
CD220	0	0	0	0	1
CD221	1	3	10	6	9
CD271	0	0	0	0	1
CD309	0	0	0	2	1
CD340	25	37	42	26	24
EGF receptor	0	0	0	1	0
fMLP receptor	0	0	0	0	0
Chemokine receptors					
CD140a	6	11	27	25	7
CD140b	98	98	99	79	95
CD183	0	0	0	1	0
CD184	0	0	0	1	0
CD193	0	11	1	9	7
CD195	0	1	0	2	2
CD196	0	2	0	0	2
CD197	0	1	0	1	1
Coagulation markers					
CD42a	0	0	0	2	1
CD42b	0	0	0	2	1
CD141	6	5	13	9	0
CD142	10	86	52	51	72
CD201	46	91	48	81	0
Tetraspanins					
CD9	94	90	68	82	54
CD37	0	0	0	1	0
CD53	0	0	0	0	0
CD63	96	98	99	89	67
CD81	100	100	100	97	100
CD151	98	99	98	94	94
CD231	0	0	0	0	0
Others					
CD26	13	72	39	65	40
CD36	0	2	3	12	1
CD57	5	1	8	2	4
CD75	0	0	0	5	3
CD76	0	0	1	5	7
CD79b	0	1	1	2	2
CD83	0	0	0	1	2
CD91	0	0	0	2	1
CDw93	0	0	0	1	3
CD99	0	6	16	33	6
CD99R	0	0	1	3	3
CD100	0	0	0	0	1
CD107a	0	2	0	9	6
CD107b	0	1	0	9	6
CD108	8	25	6	29	27
CD109	0	0	0	0	3
CD163	0	0	0	0	0
CD172b	0	0	0	0	2
CD177	0	0	0	0	1
CD205	1	3	2	5	2
CD206	0	0	0	0	2
CD335	0	0	0	0	0
CD336	0	0	0	0	0
CD337	0	0	0	1	1
GD2	24	3	21	16	6
hBLTR-1	0	0	0	0	0
CMRF-44	0	0	0	1	1
CMRF-56	0	0	0	2	1

Donor N°	89	93	98	105	107
Immune cell markers					
CD1a	0	0	0	1	0
CD1b	0	0	0	0	0
CD1d	0	1	1	1	0
CD3	0	0	0	3	1
CD4	0	0	0	2	0
CD4 v4	0	1	0	0	0
CD5	0	0	0	2	0
CD6	0	0	0	1	0
CD7	0	0	0	0	0
CD8a	0	0	0	0	0
CD8B	0	0	0	1	2
CD10	65	21	22	11	15
CD14	0	3	0	2	1
CD16	0	0	0	1	1
CD19	0	0	0	2	0
CD20	0	0	0	2	0
CD22	0	0	0	1	0
CD23	0	0	0	2	0
CD30	0	0	0	2	0
CD32	0	0	0	2	0
CD33	0	0	0	1	1
CD38	0	0	0	1	0
CD43	0	0	0	2	0
CD45	0	0	0	2	4
CD45RA	0	0	3	9	3
CD45RB	0	0	0	2	1
CD45R0	0	0	1	2	2
CD48	0	0	0	1	2
CD64	0	0	0	2	0
CD69	0	0	0	1	0
CD70	0	3	8	1	2
CD72	0	0	0	0	2
CD85j	0	0	0	1	0
CD89	0	0	0	2	1
CD94	0	0	0	2	0
CD150	0	0	0	1	0
CD152	0	0	0	2	0
CD158a	0	0	0	2	2
CD158b	0	0	0	2	3
CD161	0	0	0	1	0
CD209	0	0	0	2	2
CD229	0	0	0	1	1
CD244	0	0	0	1	0
CD305	0	0	0	1	1
CD314	0	0	0	2	1
TCR $\alpha\beta$	0	0	0	1	0
TCR $\gamma\delta$	0	0	0	1	0
Invariant NK T	0	0	0	2	1
Vbeta23	0	0	0	1	0
Vβ8 (BV8)	0	0	0	1	2
NKB1 (KIR)	0	0	0	1	0
Immune response induction/immunomodulation					
HLA-A, B, C	14	93	93	35	61
HLA-A2	0	0	0	0	0
HLA-DQ	0	0	0	2	1
HLA-DR	0	0	0	2	1
HLA-DR DP DQ	0	0	0	1	0
β2-MG	90	86	93	64	58
CD27	0	0	0	2	1
CD28	0	0	0	1	1
CD39	0	0	0	2	1
CD40	0	0	0	1	0
CD74	0	0	0	1	2
CD80	0	0	0	1	1
CD86	0	0	0	1	2
CD112	0	0	0	1	1
CD180	0	0	0	2	0
CD200	0	33	1	20	24
CD226	0	0	2	1	0
CD275	0	2	3	2	2
CD278	0	0	0	0	1
CD282	0	0	0	1	2
CD294	0	0	0	2	3
CLIP	0	0	0	1	0
MIC A/B	0	0	0	2	0
Complements					
CD21	0	0	0	1	0
CD35	0	0	0	2	1
CD46	91	96	99	90	87
CD55	49	85	83	65	60
CD59	52	99	95	91	77
CD88	0	0	0	0	0
Percentage	**0**	**25**	**50**	**75**	**100**

(a)

Figure 1: Continued.

Donor N°	89	93	98	105	107
Mesenchymal stromal cell marker					
CD73	870	1357	1536	2441	1098
CD90	974	1908	682	1351	1028
CD105	542	1882	891	2548	1306
Pluripotent stem cell markers					
CD13	1824	1759	828	2362	1927
SSEA-3	47	73	59	91	69
SSEA-4	92	76	55	104	78
TRA-1-60	38	59	44	84	72
TRA-1-81	35	60	44	82	74
Hem Pro cell	49	99	50	102	83
Adhesion markers					
CD2	36	54	46	95	68
CD11a	38	54	41	78	66
CD11b	38	58	49	91	70
CD11c	35	58	47	83	64
CD15	39	63	47	92	70
CD15s	40	60	47	86	66
CD18	34	54	45	72	62
CD24	38	56	49	84	68
CD29	786	1609	1514	1331	1462
CD31	36	64	46	93	73
CD34	42	59	45	84	72
CD41a	36	60	44	79	58
CD41b	36	59	45	81	60
CD44	220	3222	845	1430	1336
CD47	875	997	1075	1357	991
CD49a	44	236	193	227	95
CD49b	682	1220	515	2139	861
CD49c	1205	950	1695	1460	770
CD49d	73	141	138	266	226
CD49e	933	1572	1182	1421	1113
CD49f	65	68	68	79	84
CD50	43	69	54	102	99
CD51/61	77	165	138	147	133
CD54	493	476	392	1185	432
CD56	38	63	50	90	79
CD58	221	338	358	599	364
CD61	203	266	246	250	170
CD62E	38	64	44	78	73
CD62L	38	59	44	73	65
CD62P	36	63	49	91	75
CD66	42	64	45	81	70
CD66b	49	114	95	152	150
CD66f	39	65	47	88	72
CD84	42	63	44	81	65
CD97	36	56	42	79	73
CD102	35	64	49	88	69
CD103	35	58	46	91	72
CD104	38	59	49	84	79
CD106	147	74	183	120	95
CD138	35	64	45	81	64
CD144	35	61	52	86	72
CD146	480	81	330	253	103
CD147	1560	2817	1661	3039	1852
CD162	34	56	50	76	64
CD164	294	396	470	594	325
CD165	247	276	270	451	233
CD166	174	609	371	778	360
CD171	36	60	47	88	68
CD321	36	73	46	123	72
CD326	36	65	45	84	72
CDw327	32	64	40	72	60
CD328	36	65	41	88	65
CDw329	32	58	40	87	61
SSEA-1	35	59	44	86	60
CLA	42	64	59	96	73
Programmed cell death/TNF receptor superfamily					
CD95	378	828	**439**	1087	564
CD134	39	56	47	86	62
CD137 L	36	63	49	95	70
CD153	36	61	45	83	75
CD154	40	64	45	79	70
CD181	68	138	99	198	114
CD178	36	65	42	79	65
CD255	39	68	51	93	72
CD267	39	64	52	90	70
CD268	36	64	45	77	72
CD273	53	99	141	252	103
CD274	62	106	105	143	95
CD279	38	58	40	84	65
Transport protein					
CD98	895	**1930**	1612	2029	1403
CD338	38	69	46	88	74

Donor N°	89	93	98	105	107
Cytokine receptors					
CD25	40	64	52	92	70
CD120a	59	83	64	120	91
CD120b	39	64	50	102	75
CD121a	38	70	46	91	82
CD121b	38	67	46	100	74
CD122	38	63	47	93	78
CD123	38	63	46	91	74
CD124	35	58	46	90	73
CD126	32	55	45	84	78
CD127	35	56	59	93	64
CD128b	35	31	44	87	72
CD130	95	15	79	137	127
CD132	47	69	63	104	78
CD135	38	59	46	93	66
CD137	36	60	46	93	66
CD210	35	61	50	78	79
CD212	39	61	54	92	74
Hormone, growth factor, other protein receptors					
CD71	186	379	370	616	259
CD87	35	55	44	83	68
CD114	35	61	49	96	66
CD116	36	64	44	93	78
CD117	36	58	46	84	66
CD118	35	61	47	83	61
CD220	38	65	49	86	70
CD221	65	118	106	156	146
CD271	38	64	45	73	66
CD309	38	60	40	99	66
CD340	177	246	184	276	226
EGF receptor	35	65	42	87	66
fMLP receptor	34	55	41	76	61
Chemokine receptors					
CD140a	90	148	127	248	133
CD140b	866	1463	1464	1046	1046
CD183	38	63	51	81	61
CD184	38	65	50	83	70
CD193	68	127	87	191	109
CD195	42	72	55	96	78
CD196	35	61	45	78	68
CD197	36	58	49	81	66
Coagulation markers					
CD42a	36	68	44	89	73
CD42b	36	63	46	92	74
CD141	51	83	82	147	90
CD142	56	1004	219	480	654
CD201	231	662	375	689	81
Tetraspanins					
CD9	962	882	594	1125	424
CD37	36	56	47	88	65
CD53	35	59	53	83	70
CD63	757	1508	1171	1260	500
CD81	2936	2936	2936	2936	2936
CD151	848	1193	759	1793	1076
CD231	35	64	45	84	64
Others					
CD26	77	526	134	745	260
CD36	48	69	60	134	78
CD57	113	91	83	147	112
CD75	38	68	49	108	75
CD76	31	56	74	179	94
CD79b	35	67	49	83	70
CD83	39	65	45	83	69
CD91	38	68	45	96	74
CDw93	36	69	51	92	79
CD99	53	120	114	291	114
CD99R	35	68	46	101	80
CD100	38	61	46	86	68
CD107a	48	76	45	74	92
CD107b	58	78	46	81	100
CD108	79	140	67	232	198
CD109	36	63	47	86	75
CD163	36	64	51	86	62
CD172b	38	67	49	88	72
CD177	36	61	42	79	64
CD205	58	101	76	178	92
CD206	35	58	45	81	70
CD335	32	59	44	79	62
CD336	32	54	44	70	58
CD337	38	56	40	87	69
GD2	86	61	82	148	74
hBLTR-1	36	59	42	82	62
CMRF-44	35	59	45	77	64
CMRF-56	35	64	41	88	66

Donor N°	89	93	98	105	107
Immune cell markers					
CD1a	36	54	41	92	64
CD1b	36	59	45	79	58
CD1d	47	73	54	91	69
CD3	40	56	46	91	65
CD4	36	61	51	105	62
CD4 v4	40	65	51	91	66
CD5	36	58	42	92	65
CD6	34	55	41	91	61
CD7	32	60	46	80	62
CD8a	35	52	42	82	58
CD8B	36	63	45	91	74
CD10	346	141	79	148	147
CD14	35	63	46	81	68
CD16	38	59	46	77	65
CD19	36	60	47	91	65
CD20	36	56	44	90	70
CD22	36	55	41	74	62
CD23	36	58	49	87	69
CD30	39	63	49	81	68
CD32	38	60	46	93	81
CD33	36	59	44	79	70
CD38	38	59	45	78	58
CD43	40	65	46	87	72
CD45	36	59	47	93	100
CD45RA	51	84	64	127	90
CD45RB	36	58	45	84	70
CD45R0	39	60	46	81	70
CD48	39	65	49	90	72
CD64	34	58	45	79	65
CD69	35	63	47	83	66
CD70	40	77	56	99	75
CD72	38	64	44	102	82
CD85j	35	58	44	79	60
CD89	34	65	45	90	72
CD94	38	56	46	90	69
CD150	39	64	52	82	70
CD152	36	60	45	83	66
CD158a	38	63	47	88	72
CD158b	35	64	51	84	72
CD161	35	59	50	76	69
CD209	42	68	54	95	79
CD229	35	56	49	87	60
CD244	35	63	46	83	68
CD305	34	52	41	72	58
CD314	36	61	41	88	64
TCR $\alpha\beta$	35	59	45	79	64
TCR $\gamma\delta$	34	52	41	69	60
Invariant NK T	35	56	44	86	61
Vbeta23	36	60	44	79	65
Vβ8 (BV8)	43	69	51	87	68
NKB1 (KIR)	35	59	41	87	60
Immune response induction/immunomodulation					
HLA-A, B, C	135	888	690	306	455
HLA-A2	36	61	44	79	61
HLA-DQ	38	58	42	81	58
HLA-DR	38	65	47	93	68
HLA-DR DP DQ	35	61	42	87	65
β2-MG	578	678	704	771	504
CD27	36	63	45	88	68
CD28	38	58	47	86	68
CD39	39	65	49	92	68
CD40	38	56	49	84	61
CD74	39	60	44	71	70
CD80	40	58	44	76	65
CD86	32	64	40	82	78
CD112	34	54	45	73	70
CD180	38	64	46	90	69
CD200	35	152	46	181	126
CD226	36	60	54	74	64
CD275	42	70	60	88	74
CD278	32	60	45	69	62
CD282	42	54	40	76	64
CD294	38	63	54	97	75
CLIP	36	58	44	79	61
MIC A/B	35	69	44	90	65
Complements					
CD21	38	58	42	82	65
CD35	38	58	49	91	78
CD46	467	1090	767	1320	686
CD55	242	618	398	686	454
CD59	246	1101	557	1181	636
CD88	32	59	41	77	65
MFI	**50**	**250**	**500**	**750**	**1100**

(b)

Figure 1: Heat map of cell surface marker expression of ADHLSCs using the BD Lyoplate. Results are expressed as percentage of positive cells (a) and in median fluorescence intensity of the total population (MFI) (b). For reference, isotype controls showed an average MFI of 60 ($n = 5$).

marrow derived MSCs (CD34, CD36). ASCs also express CD91 which is absent from ADHLSCs (Table 2).

ADHLSCs did not appear to express markers of pluripotent stem cells, except for CD13, which was expressed at very high levels, confirming our previous results (Figure 1) [2]. However, it should be noted that, despite CD13 being initially described in relation to pluripotent stem cells, subsequent studies suggested an additional role for the molecule, including cell adhesion [16, 17]. Interestingly, it has been implicated in the adhesion of monocytes to the endothelium.

3.2. Immunogenic Phenotype. ADHLSCs did not express any immune cell markers, as expected. However, these cells did express human leukocytes antigens (HLA) A, B, and C and β2-microglobulin, which are components of MHC class I, as do all nucleated cells, and could therefore be the target of cytotoxic T cells (Figure 1). Moreover, ADHLSCs did not express any of the other proteins tested that could trigger an immune response during infusion (Figure 1). These results seem to be in accordance with our previous reports that ADHLSC are poorly immunogenic [6]. In fact, our data suggest that these cells are immunosuppressive. However, the list of immunomodulatory markers tested here is not exhaustive and the immunosuppression assays performed were limited to the inhibition of PHA/IL-2 stimulated T cells. Therefore, further research would have to be performed to confirm the poorly immunogenic phenotype of ADHLSCs and better understand their effect on immune cells. In addition, our results suggest that ADHLSCs could be protected against the complement cascade following infusion thanks to the expression of CD46 and CD55, which may inactivate proteins C3b and C4b, and the expression of CD59, which can block complement protein C9. Nevertheless, expression of CD95 (Fas receptor) by ADHLSCs renders cells susceptible to apoptosis through ligation by a secreted Fas ligand protein or contact with a Fas ligand-bearing adjacent cell [18].

3.3. Procoagulant Phenotype. ADHLSCs have been shown to have procoagulant activity due to the presence of tissue factor (CD142) [19], a member of the coagulation cascade required for thrombin formation, expression of which we confirm in this study. We also show that ADHLSCs do not express CD42 (a or b), which are platelet surface glycoproteins, or CD141 (thrombomodulin). They do, however, express CD201, also known as activated protein C receptor, which plays a role in anticoagulation (Table 2). It is noteworthy that the level of expression of CD141 and CD142 varied from donor to donor, suggesting that the pro- or anticoagulant properties of ADHLSCs are likely to be donor-dependent.

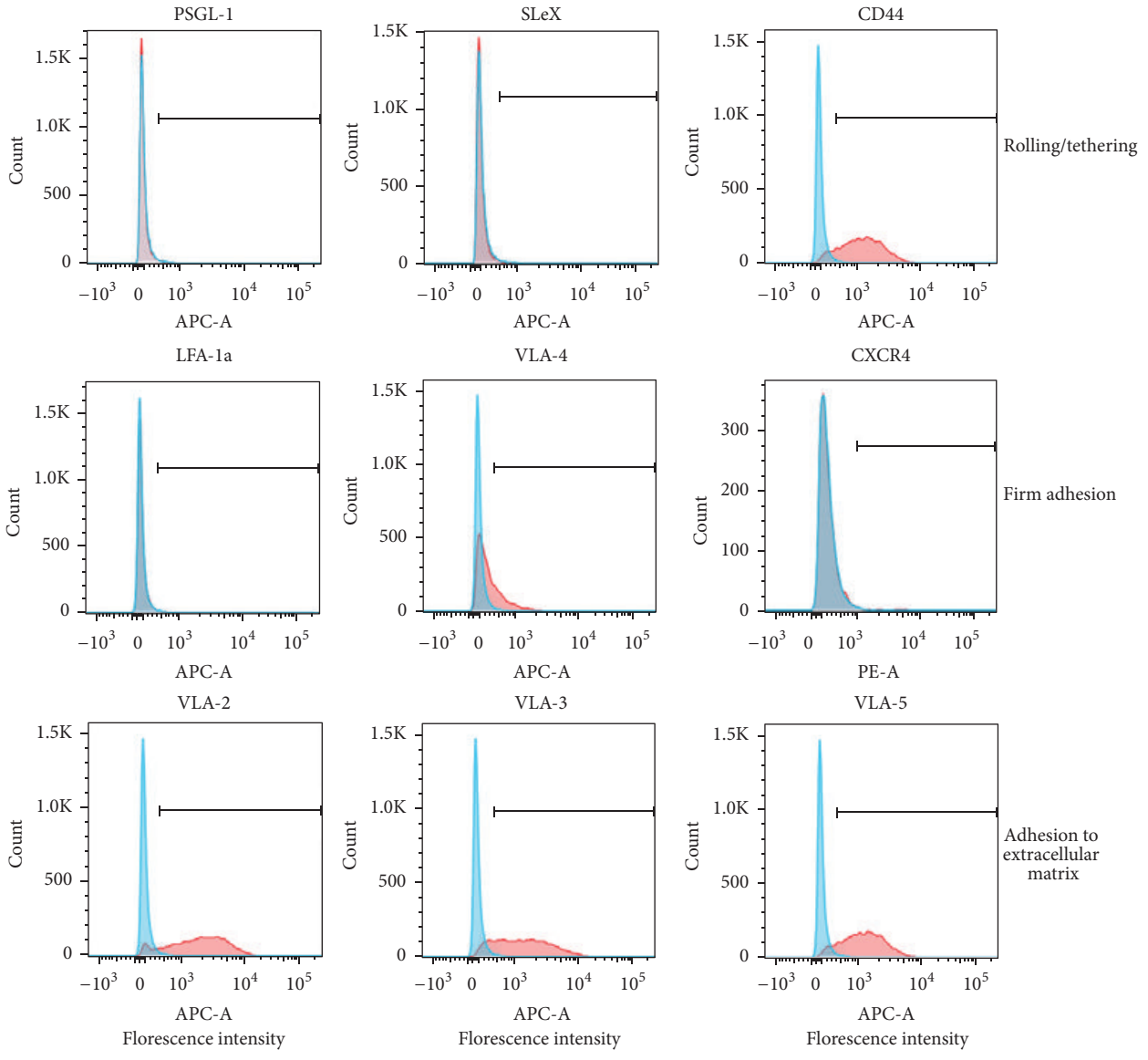
3.4. Tetraspanin, Cytokine, Chemokine, Hormone, and Growth Factor Receptor Expression. Our study shows that ADHLSCs express several tetraspanin family members, such as CD9, CD63, CD81, and CD151. Although their function is not entirely known, they appear to play a role in signal transduction. A transporter of amino acids (CD98) was also detected, as was the DPPIV enzyme, also known as CD26, which is highly expressed in the liver [20].

Only a few cytokine, chemokine, hormone, and growth factor receptors were found. CD71 (transferrin receptor protein 1) and CD140b (beta-type platelet-derived growth factor receptor) were detected on the surface of ADHLSCs at passage 5 (Table 2). However, it is possible that some receptors became internalized during the culture process.

3.5. Adhesion Proteins. This study was designed in part to evaluate the expression of adhesion proteins that would allow ADHLSCs to bind to the endothelium and extracellular matrix during the engraftment process. In order to reach the parenchyma of the organ following peripheral injection, MSCs must behave like leukocytes during inflammation: first, they must decrease their speed on the endothelium with the help of selectin ligands; second, they must adhere firmly to endothelial proteins such as ICAM and VCAM-1 using integrin dimers like VLA-1 (αLβ2) and VLA-4 (α4β1), respectively. BD Lyoplate screening was followed by evaluation of the expression of some of the proteins of interest at the mRNA level using the TaqMan array for human extracellular matrix and adhesion molecules. This was done in order to distinguish molecules that are completely absent from those that are absent or barely expressed at the protein level, but expressed at the mRNA level. As shown in Figures 1 and 2, ADHLSCs, like most MSCs, did not express either CD162 (PSGL-1) or sialyl-Lewis X (SLeX), a tetrasaccharide component of PSGL-1 required to bind E-selectin, on their surface. Real-time PCR analysis proved that they were not expressed at the mRNA level either. Sarkar et al. were able to make MSCs roll on P-selectin and activated endothelial cells by linking a sialyl-Lewis X group to the cell surface via a biotin streptavidin bridge, thereby decreasing the rolling velocity of cells in vivo [11]. Interestingly, CD44, which is considered by some [8, 9] as an alternative for the rolling/adhesion process, is highly expressed by ADHLSCs. However, in these publications, CD44 had to be engineered with the fucosyltransferase enzyme to have the ability not only to bind selectins, but also to improve cell engraftment of BM-MSCs in NOD/SCID mouse bone marrow after 24 hours [21, 22]. Because fucosyltransferase IV (SSEA-1) is not expressed by ADHLSCs (Figure 1) [21], CD44 may not be functional as an adhesion protein under noninflammatory conditions [21]. However, cell adhesion may depend on the organ targetted and its inflammatory status. Indeed, reports have shown that while neutrophils rely on tethering and rolling followed by firm adhesion to integrate most tissues, in the inflamed liver, their adhesion to the sinusoidal endothelium relies on direct binding of their CD44 to the hyaluronan expressed by the endothelial cells [23, 24]. Therefore, the lack of PSGL1 and other adhesion molecules involved in rolling and firm adhesion may be overcome by the high expression of CD44 at the surface of ADHLSCs. In addition, binding to hyaluronan in the sinusoids may help keep the cells in the liver and limit their dissemination to nontargetted organs. Our results also reveal that ADHLSCs, like all MSCs, do not express VLA-1 at the protein or mRNA level, but they do show a slight expression of VLA-4 on their surface (average MFI of 168.8 for 5 donors) (Figures 1 and 2) [12, 25],

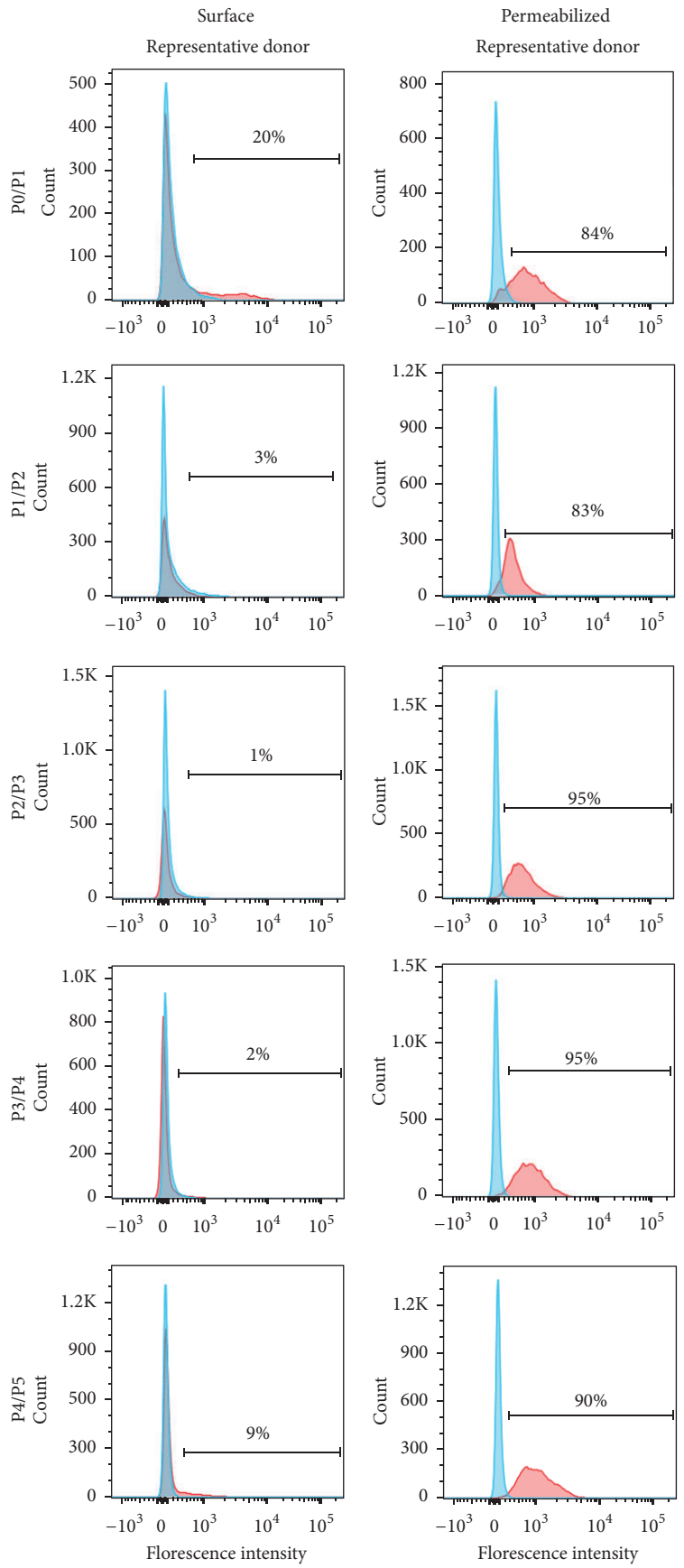
	Protein expression (FACS, MFI, $n = 5$)	mRNA expression (PCR, Ct, $n = 4$)
PSGL-1	Negative (57 ± 7)	Undetermined
SLeX	Negative (61.8 ± 9)	N.D.
LFA-1	Negative (55.4 ± 7.5)	Undetermined
VLA-2	High (1083.4 ± 288.6)	Positive (Ct: 29.6 ± 2.8)
VLA-3	High (1216 ± 167)	Positive (Ct: 26.6 ± 3.7)
VLA-4	Low (168.8 ± 34.4)	Positive (Ct: 29.3 ± 2.7)
VLA-5	High (1244.2 ± 113.3)	Positive (Ct: 24.8 ± 3.2)
CD44	High (1410.6 ± 501)	Positive (Ct: 25 ± 2.7)
CXCR4	Negative (61.2 ± 7.8)	Positive (Ct: 34.3 ± 2.2)

(a)



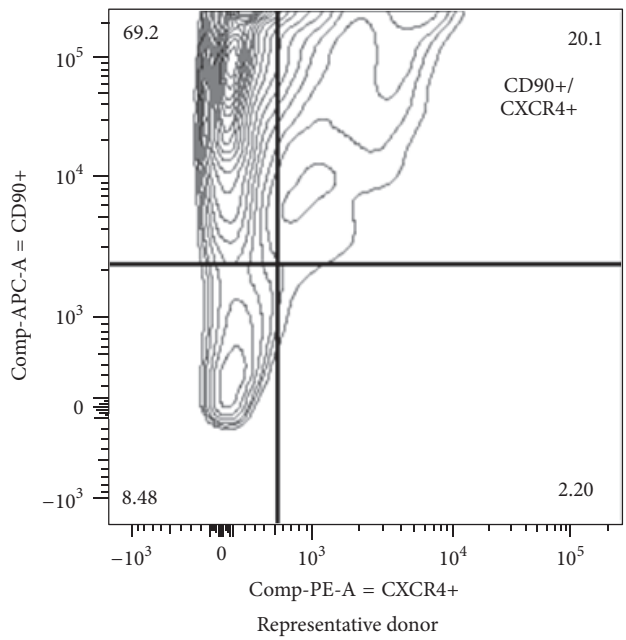
(b)

Figure 2: Antigen and mRNA expression of the main molecules involved in the engraftment process. (a) Results are expressed in mean (± standard error of the mean) of median fluorescence intensity for the protein expression (FACS; $n = 5$ donors) and of Ct values for the mRNA expression (real-time PCR; $n = 4$ donors). (b) Histograms of the antigens PSGL-1, LFA-1α, VLA-4, and VLA-5; analyses with FlowJo. Red histograms represent cells stained for the antigen of interest versus cells stained with appropriate isotype controls in blue.

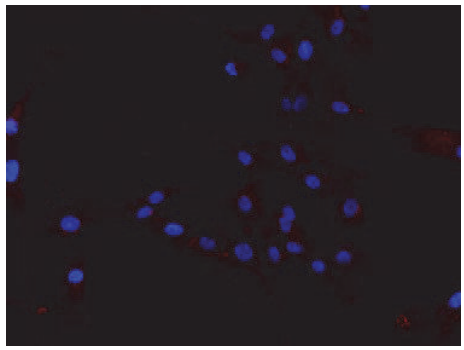


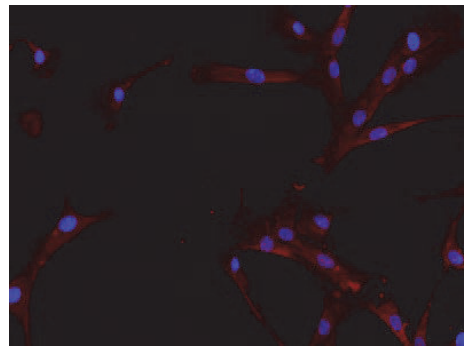
(a)

Figure 3: Continued.



(b)





(c) (d)

Passages	P0/P1	P1/P2	P2/P3	P3/P4	P4/P5
Surface	23.1% ± 5.3%	13.3% ± 9.9%	1.5% ± 0.3%*	2.1% ± 0.5%*	9.7% ± 3.7%
Permeabilized	80.3% ± 1.5%	82.7% ± 6.6%	85.7% ± 0.3%	94.7% ± 1%	90.9% ± 3.9%

(e)

Figure 3: CXCR4 expression of ADHLSCs. (a) CXCR4 surface and internalized expression from passages 1 to 4 by flow cytometry. (b) Contour plot of double staining for CD90 and CXCR4 at passage 1. Representative immunofluorescence pictures of CXCR4 staining at passage 4 for permeabilized (d) and nonpermeabilized (c) ADHLSCs. (e) Cell surface and intracellular CXCR4 expression (± standard error of the mean) from passages 1 to 5 ($n = 4$ donors); significant differences have been found on the surface expression between P0/P1 and P2/P3 and P3/P4 ($^*P < 0.05$).

despite a constitutive expression at the mRNA level (Figure 2). In addition, ADHLSCs show high expression of all the integrins needed to bind to the extracellular matrix once they have passed through the endothelium: VLA-2 to bind to collagen, VLA-3 to bind to laminin, and VLA-5 to bind to fibronectin [26]. However, even if ADHLSCs express most of the integrins needed to bind to the extracellular matrix, low expression of VLA-4, which appears to be the most important protein for the rolling/adhesion process and binding to the endothelium, and the absence of selectin ligand may be sufficient to explain the low engraftment rates, as cells are unable to stop and attach to the endothelium [12, 27]. On the other hand, upregulation of integrin alpha 4 by an adenovirus vector was shown to increase cell engraftment by 25% in the bone marrow of C57BL mice [28]. Further research is currently under way to determine the importance of these molecules in the adhesion of ADHLSCs to the endothelium and the extracellular matrix.

3.6. CXCR4 Expression. Another important protein involved in the homing process is the 7-transmembrane G-coupled receptor CXCR4. CXCR4 has been described as the major receptor involved in the engraftment/homing process of HSCs and MSCs. At injury sites, CXCR4 binds released SDF-1, which facilitates cell migration to organs [13]. Use of the CXCR4 antagonist AMD3100 during cell infusion was shown to inhibit migration of MSCs to the acutely injured kidneys [29, 30]. However, several groups have reported that CXCR4 expression decreases rapidly after MSCs isolation and only a very small percentage of cells or none at all express CXCR4 after a few passages [31, 32]. In fact, in vitro expansion of MSCs induces progressive internalization of CXCR4 as a way for cells to adapt to culture conditions, to the point where there is no CXCR4 remaining on their surface [12, 33–35]. Considering that ADHLSCs emerge from the parenchymal fraction of the adult liver after about one month in culture and then need several more weeks to reach passages 4 to 6 at which time they are traditionally used for experiments, we suspected that the same phenomenon may be taking place. We therefore evaluated surface expression of CXCR4 at each passage by flow cytometry and found that all donors tested showed a surface receptor expression around 23.1% ± 5.3% at passage 1 (Figure 3(e)), which decreased significantly until 2.1% ± 0.5% at the fourth passage. However, when the cells were permeabilized, 80.3% ± 1.5% of the cell population expressed CXCR4 at passage 1, and the expression remained at 94.7% ± 1% at passage 4, suggesting that a large portion of the population had already started to internalize CXCR4 ($n = 4$). To verify that the CXCR4-positive cells were indeed ADHLSCs, double staining of CXCR4 with CD90 was performed on three donors (Figure 3(b)). These results show that ADHLSCs have a pattern of CXCR4 expression identical to that of BM-MSCs, which could be a specific characteristic of MSCs. Rombouts and Ploemacher found that the engraftment capacity of freshly isolated MSCs was higher than that of cultured MSCs even after only 24 hours of culture [36]. Consequently, some research groups have decided to induce externalization of CXCR4 on the surface of MSCs, a key point to enhance MSC homing. Different methods have been used to upregulate CXCR4, such as culture with valproic acid (VPA) [37, 38], C1q [39], SDF-1 [40], or a cytokine cocktail [34], culture under hypoxic conditions [41], or even direct transduction with a gene encoding the receptor [42]. In our experience, neither the cocktail of cytokines described by Shi et al., nor preincubation of ADHLSCs with SDF-1 [40] had any effect on CXCR4 externalization. However, in a patient with factor VIII deficiency infused intravenously with ADHLSCs at passages 4 and 5, we found cells engrafted at the injury site (hemarthrosis), highlighting the capacity of ADHLSCs to engraft into inflammatory areas (unpublished data).

4. Conclusion

In conclusion, even if we cannot completely rule out that the low levels of engraftment observed following infusion are due to cell clearance, our data suggest that ADHLSCs are poorly immunogenic overall. However, adhesion molecules expressed by the cells appear to point to an impaired capacity to bind to the endothelium, which could potentially lead to lower engraftment. In addition, our findings indicate that the pattern of expression of some of these proteins may result from the culture process. Further studies are needed to evaluate the precise impact of culture conditions on the expression of cell surface markers by ADHLSCs and determine whether modifying the culture process could improve cell engraftment.

Competing Interests

The authors declare that there are no competing financial interests.

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