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LETTER TO THE EDITOR

Aberrant expression of HLA-DR antigen by bone marrow-derived mesenchymal stromal cells from patients affected by acute lymphoproliferative disorders

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Mesenchymal stromal cells (MSCs) can be isolated from various sources and their plasticity and multilineage potential to differentiate into other mesodermal cells has been widely reported.[1–2] Host immune response has been the subject of numerous recent studies, in which it was demonstrated that MSCs have an immunomodulant role.[3] Human MSCs have been shown to escape recognition by alloreactive T cells in mixed lymphocyte reaction cultures (MLR).[3] The evidence that *in vitro* culture-expanded MSCs can be infused intravenously without toxicity have suggested that allogeneic MSC have many advantages in therapeutic applications such as enhancement of hematopoietic stem cells engraftment and reduction of the incidence and severity of graft-versus-host disease.[4] The immunosuppressive nature of differentiated and undifferentiated bone-marrow (BM)-MSCs could also be of clinical importance in the treatment of autoimmune diseases.[4] However, the mechanisms by which MSCs can exert their immunomodulant role are under investigation.[5] Moreover, a few data are available in the literature regarding the heterogeneous pattern of response in inhibiting T-cell proliferation by cultured BM-MSCs from patients with hematological malignancies.[6]

In this study, the expression of human leukocyte antigen (HLA), major histocompatibility complex (MHC) class I and II were investigated on *in vitro* cultured BM-derived MSCs, based on the notion that either undifferentiated or differentiated MSCs express intermediate levels of HLA class I, but do not express the class II molecules.[7] However, it is known that HLA-DR expression by MSCs can be induced by stimulation with interferon γ. Interestingly, both undifferentiated and differentiated MSCs do not express costimulatory molecules such as B7-1, B7-2, CD40 or CD40L and fail to elicit proliferation of allogeneic lymphocytes.[7]

In brief, BM-derived MSC were isolated from eight normal subjects (NS-MSC), (mean age: 52±8, two male subjects, six female subjects), 13 subjects affected by lymphoprolipherative disorders (LPD-MSCs), eight non-Hodgkin's lymphoma (NHL) showing BM-infiltration (one of whom having a Burkitt type NHL: mean age 54±12, five male subjects and three female subjects); five acute lymphoblastic leukemia (ALL) L3 subtype: n. 3, L2 subtype: n. 2 (precursor B-cell ALL) (mean age 31±18, three female subjects, two male subjects). MSCs from other sources such as skin (SF) and adipose tissue (AT) were also tested for comparison. The isolation, and the *ex vivo* expansion protocols of MSCs were based on previously published reports.[7,8] In this study, BM-MSCs were isolated and cultured using two different media: Dulbecco's Modified Eagle medium (DMEM-LG) (Invitrogen Ltd, Paisley, UK; Invitrogen-Gibco, Milan, Italy), in addition with 10% of FBS (fetal bovine serum, StemCell Technologies Inc., Vancouver, BC, Canada) and 1% of antibiotic-antimycotic and 1% L-glutamine (Invitrogen Ltd, Paisley, UK) (M^{LB}) as reported by Le Blanc *et al.*[7] and the commercially available endothelial growth medium (EGM)-2 Bullet kit with basal medium and cytokine's SingleQuots: 2% of FBS, human-fibroblast growth factor (hFGF), vascular endothelial growth factor, R3-insulin like growth factor, human-epidermal growth factor (hEGF), GA-1000, heparin, hydrocortisone, ascorbic acid (M^{EGM}) (Biowhittaker, Cambrex Bio Science Walkersville Inc., USA). After 20 days of culture, MSCs from all sources were detached and their immunophenotypic profile was firstly analyzed at first passage by using different monoclonal antibodies (MoAbs) against HLA-DR, class II molecule: HLA-DR (Tu36, immunoglobulin (IgG2b), fluorescein isothiocyanate (FITC), phycoerythrin (PE), PE-Cy5.5, Caltag, Laboratories) and HLA-DR (clone L243, IgG2a, FITC, PE, PE-Cy5.5, from BD PharMingen, San Jose, CA, USA), and anti-HLA-ABC (Tu149, IgG2a, FITC), and CD80 (MEM-233, IgG1,PE) MoAbs. To characterize MSCs, we also used a wide panel of MoAbs directed against adhesion molecules (CD29, CD106, CD105, CD166, CD36), extracellular matrix proteins (CD90, CD44),

Table 1 The mean percentage of HLA-DR and CD80 expressing LPD-MSCs are reported in relation to the different time points of the culture (days), the *in vitro* culture conditions (LB vs EGM medium) and the different MSCs morphological characteristics (R1 vs R2 gates)

Morphological gate on BM-MSCs	M^{LB} 20days percentage of positive MSCs (gated on R1–R2 CD45-/7-AAD- events)		M^{LB} 50days percentage of positive MSCs (gated on R1–R2 CD45-/7-AAD- events)		M^{EGM} 20days percentage of positive MSCs (gated on R1–R2 CD45-/7-AAD- events)		M^{EGM} 50days percentage of positive MSCs (gated on R1–R2 CD45-/7-AAD- events)	
	R2	R1	R2	R1	R2	R1	R2	R1
LPD-MSCs (23%)[a]								
HLA-DR+	5±4	20±3	0	0	41±8	50±7	17±11	19±14
HLA-DR+/CD80+	0	0	0	0	6±7	5±9	0	0

Abbreviations: BM, bone marrow; HLA, human leukocyte antigen; LPD, lymphoprolipherative disorder; MSC, mesenchymal stromal cell.
Four-colors flow cytometric analysis: CD45-/7-AAD- MSCs were gated on the basis of light scattering properties (R1 large hypergranular cells; R2 small hypogranular cells). Results obtained from the study of SF, AT and BM-derived NS-MSC were not included in the table, as they resulted HLA-DR and CD80 negative.
Data are expressed as mean±s.d.
[a]% of patients showing HLA-DR+ MSCs.

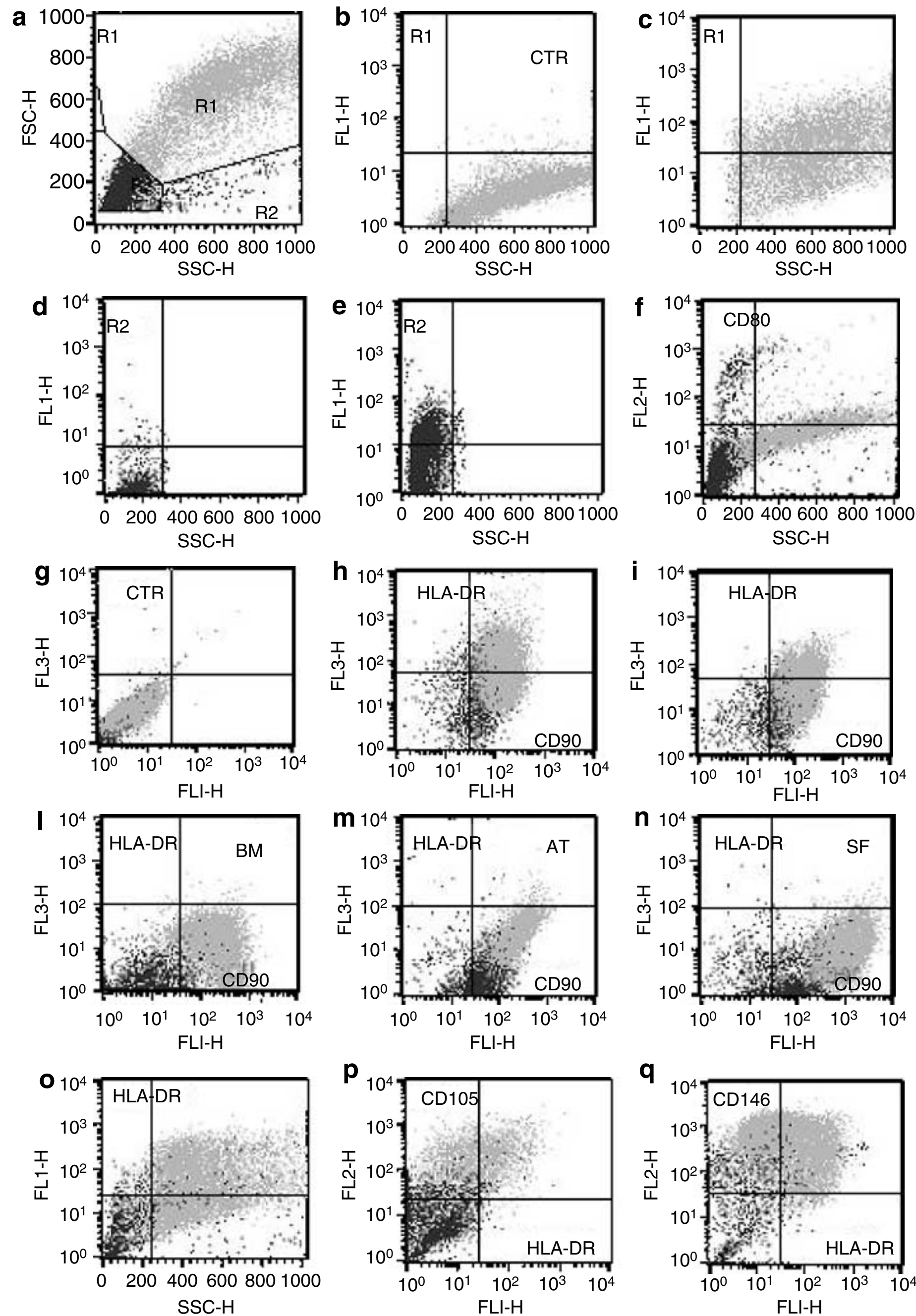

Figure 1 The flow cytometric analysis of HLA-DR on LPD-MSC, cultured in M^{EGM} medium, are reported. (**a**–**f**) Cell positivity for HLA-DR molecule was assessed on two MSCs sub-populations, as identified by morphological characteristics (R2, small hypogranular cells; R1, large hypergranular cells). (**a**–**e**) Both MSC cell subsets (R1 and R2) resulted HLA-DR+ in MHC class-expressing cases. (**g**–**i**) HLA-DR expression on cultured LPD-MSCs was decreased with increasing age of the culture (G is the isotypic control, H at 18 days, I at 40 days of culture). (**l**–**m**) HLA-DR expression resulted negative in BM-MSC from normal controls, from AT and on skin fibroblast (SF). (**o**–**q**) HLA-DR is expressed on different LPD-MSC immunologic subsets.

hematopoietic markers (CD31, CD34, CD11c, CD14, CD45-leukocyte common antigen), and complement regulatory proteins (CD59).[8] The analysis of MSCs was performed using a multicolor flow cytometric protocol based on the exclusion of CD45+ (known to be a hematopoietic marker) and 7-AAD+ (a nucleic acid staining compound, used for the recognition of dead and apoptotic cells) events. Flow cytometry analysis was also performed on MSCs taken at second and third passage of culture and obtained from the two different culture conditions.

In order to test the MSCs immunosuppressive properties, *in vitro* MLR (with and without mesenchymal cells) were assessed as described previously.[3,7]

Our results showed that BM-MSCs were positive for CD90, CD105, CD44, CD29, CD146, HLA-ABC class I antigens,[8] and negative for CD45, CD34, CD14 hematopoietic markers. Interestingly, 40% of patients with ALL and the Burkitt variant of NHL had an aberrant expression of HLA-DR on cultured MSCs. Briefly, the mean percentage of HLA-DR positive LPD-

MSCs cultured with standard media (M^{LB}) was significantly higher (23% ± 22 s.d.; $P<0.05$) than that of cultured NS-MSCs (at 20 days of culture), SF and AT, being the latter always HLA-DR negative (Table 1). Interestingly, the percentage of the LPD-MSCs expressing HLA-DR was significantly increased (55% ± 32 s.d.), when cultured for 20 days in M^{EGM} angiogenic culture medium. As showed in Table 1, this upregulation was particularly evident on R2-gated $CD45^{neg}$/7-AAD^{neg} MSCs (R2 identifies smaller and hypogranular MSCs) from LPD patients. No differences in HLA-DR antigen expression were found using different anti-HLA-DR MoAbs or varying fluorochromes. Furthermore, the HLA-DR expression on cultured LPD-MSCs decreased with increasing age of the culture (>40 days) and resulted negative in normal controls, AT and SF. Concerning the immunosuppressive properties of LPD-MSCs, we showed that *ex vivo* expanded HLA-DR expressing -MSCs showed a variable *in vitro* immunosuppressive function as assessed by calculating the percentage of inhibition of T-cells proliferation in MLR cultures (range of inhibition 15–40%). Interestingly, although CD80 costimulatory molecule is reported to be constantly absent in human MSCs, we observed it expression on MSCs taken from only one case with LPD (Figure 1). In this case, HLA-DR+/CD80+ cultured MSCs showed a better capacity to elicit T-cells proliferation (percentage of lymphoproliferative inhibition observed in MLR performed in the presence of MSCs: 15%).

In recent years, MHC abnormalities such as a defect in HLA-DR expression was reported in NHL cells,[9] or in thymocytes obtained from Hashimoto disease even if this expression is not sufficient to initiate autoimmunity.[10] Stromal cells are generally believed to be responsible for establishing the thymic microenvironment, and consequently, for inducing the steady state production of mature T lymphocytes from uncommitted progenitors thus revealing important principles about the lymphoid differentiation process.[11] In our study, an aberrant HLA-DR expression on BM-derived MSCs was documented in some LPD, thus suggesting unreported possible interactions between BM stromal cells and early lymphoid progenitor cells. Furthermore, because cytokines treatment (IL-1b, INF-y and TGF-β) was reported to modulate the expression of antigen-presenting molecules on cultured human fibroblasts and epithelial cells,[12] in this study, we gave evidence that HLA-DR expression on MSCs was also found to be related to different culture conditions,[13] as it was more expressed under angiogenic conditions. As MSCs heterogeneity could suggest specialization of function, the occurrence of immunophenotypic changes of *ex vivo* expanded MSCs could be relevant for MSCs properties; based on these considerations, we speculated that the BM microenvironmental conditions play a pivotal role in controlling MSCs function. As in this study BM-MSCs were treated with angiogenic cytokines, with the aim of mimicking the BM angiogenesis process,[14] it could be argued that HLA-DR upregulation on MSCs from LPD could be a first step towards *in vitro* endothelial commitment. However, under our experimental conditions prolonged angiogenic treatment of cultured CD105+CD146+ LPD-MSCs never induced the expression of other specific endothelial markers such as CD31 and CD34.

In conclusion, the exact biological significance for the expression of class II HLA-DR molecule on BM LPD-MSCs is not fully elucidated. However, the data here presented may have therapeutic implication, as the use of autologous MSCs has been proposed in a wide range of clinical applications, including those in the area of the regenerative medicine and cell therapies.

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