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Article

Inactivated Platelet Lysate Supports Proliferation and Immunomodulant Characteristics Of Mesenchymal Stromal Cells in GMP Culture Condition

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Abstract:

For their clinical use Mesenchymal Stromal Cells (MSCs), isolated from bone marrow (BM-MSCs) are considered Advanced Therapy Medicinal Products (ATMP) and need to be produced according to Good Manufacturing Practice (GMP). Human platelet lysate (HPL) represents a good GMP-compliant alternative to animal serum and we demonstrated that after pathogen inactivation with Psoralen , it was more efficient and safer to produce MSCs in GMP. In this study MSCs cultivated in FBS (FBS-MSC) or inactivated HPL (iHPL-MSC), were compared for their immunomodulant properties. In particular, the effects of MSCs on: 1)proliferation of total Lymphocytes (Ly) and on naïve T Ly subsets induced to differentiate versus Th1 and Th2 Ly; 2) the immunophenotype of different T cell subsets; 3)the cytokine release to verify Th1, Th2 and Th17 polarization were analyzed by using in vitro co-culture system. We observed that iHPL-MSCs showed the same immunomodulant properties observed in the FBS-MSCs co-cultures. Although, a more efficient effect on the increase of naïve T cells and, in the Th1 cytokine release related to iHPL was observed. This study confirms that iHPL, used as medium supplement, may be considered a good alternative to FBS for a GMP-compliant MSC expansion to preserve also their immunomodulant proprieties.

Keywords: Mesenchymal Stromal Cells; Good Manufacturing Practice; Inactivated Platelet Lysate

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1. Introduction

Mesenchymal stromal cells (MSCs) are multipotent cells that can be isolated from a variety of tissues, capable of producing important factors including multiple cytokines and growth factors. In the last years their peculiar immunomodulant characteristics, mediated by the release of a plethora of trophic factors in their secretome, have been considered more than their multilineage differentiation potential for their clinical use in clinical trials in severe disease: autoimmune, chronic inflammatory and degenerative conditions [1,2].

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For their clinical use MSCs, isolated from bone marrow (BM-MSCs) are considered Advanced Therapy Medicinal Products (ATMP) and need to be produced according to Good Manufacturing Practice (GMP) [3,4]. Since the use of xenogeneic protein free GMP-compliant growth media is a prerequisite for clinical MSC isolation and expansion, human platelet lysate (HPL) has been efficiently substituted to fetal bovine serum (FBS) into MSC clinical manufacturing. For these reasons, it represents a good GMP-compliant alternative to animal serum for MSC clinical production confirming recent data reported in the literature [5–7]. As the risk of transmission of infective agents not routinely tested, or for which no tests are available remains, HPL quality and safety had to be greatly improved. We demonstrated that pathogen inactivation with Psoralen was efficient to isolate and expand safer MSC in GMP conditions [8]. Pathogen Inactivation (PI) technology was used for the first time in 1991 [9] to treat fresh-frozen plasma and then also for platelet and red cells (RBCs) [10]. This technology efficiently remove a wide range of pathogens, with no toxicity or effect on product potency and might also prevent the transmission of unknown pathogens [11–15]. For this reason to make a safer preparation of HPL for GMP production we used pathogen inactivation by psoralen to make HPL safer for the production of MSCs in GMP condition [8]. We also demonstrated that HPL subject to pathogen inactivation (inactivated HPL: iHPL) was more advantageous in terms of cellular growth and stemness in MSCs isolated from bone marrow (BM-MSCs). On the base of our findings about iHPL and of literature data on a possible decrease of immunomodulant properties of MSCs cultured in HPL [16] we studied some iHPL effects on immunomodulatory properties of MSCs.

In this study BM-MSCs were isolated and expanded simultaneously in iHPL (iHPL-MSCs) in GMP compliant conditions and in FBS (FBS-MSCs) in standard condition usually reported in the literature in the immunomodulant studies [17]. In particular, we focused on the effects of FBS-MSCs and iHPL-MSCs on T lymphocytes (Ly) as investigated also in a previous paper [18]. MSCs do not express MHC class II and costimulatory molecules, such as CD40, CD80 or CD86, and different studies show that MSCs are able to inhibit or limit inflammatory responses and mitigate anti-inflammatory pathway inhibiting directly or indirectly disease-associated Th1, Th2, and Th17 cells as well as cytotoxic T lymphocytes [17–20]. The effects of MSCs cultivated with the two different supplements were analyzed by using in vitro co-culture system with Peripheral Blood Mononuclear Cells (PBMC) stimulated with Phytohemagglutinin (PHA-PBMC). In particular, the following points were analyzed:

- the effect on proliferation of total Ly;
- the effect on proliferation of naïve T Ly subsets induced to differentiate versus Th1 and Th2 Ly;
- the immunophenotype of different T cell subsets (naïve, memory, effector, Th1 and Th2 lymphocytes);

2. Experimental Section

2.1. Isolation and expansion, analysis and characterization of human MSCs

Human BM samples were obtained from healthy donors from the discarded collection bag, after filtration of BM collection performed for a familiar allogeneic hematopoietic stem cell transplantation. Informed written consent was obtained from each donor in accordance with the Declaration of Helsinki and the City of Health and Science of Turin - Ordine Mauriziano hospitals' ethics committee.

The bag was washed 3 times with Phosphate Buffer Saline (PBS) 1X (Lonza, Versviers, Belgium) and the cells were collected and washed at 200g for 10 minutes. An aliquot of whole BM was counted and plated directly in T75 o T150 flasks (Becton Dickinson, Franklin Lakes, NJ, USA) at $1x10^4$ cells /cm^2. The cells were simultaneously plated in two different condition containing α MEM (Biochrome, Berlin, Germany), 2mM L-glutamine (Sigma-Aldrich®) and penicillin/ streptomycin 1X (Euroclone, Pero, Mi, Italy) supplemented with 10% of FBS (Sigma-Aldrich®) or 10% of iHPL. HPL was prepared in the Blood Component Production and Validation Center, City of Health and Science of Turin, S. Anna Hospital, from a pool of platelets of healthy donors and subjected to pathogen inactivation by psoralen as described in [21].

The culture was maintained at 37°C with a 5% CO^2 atmosphere. After 5-7 days, the non-adherent cells were removed, and the adherent cells were re-fed every 3-4 days. In order to expand the isolated cells, the adherent semi-confluent monolayer was detached with trypsin/EDTA 1X (Sigma-Aldrich®) for 5 minutes at 37°C and expanded for several passages until they no longer reached confluence.

Only the cells which were compliant to the International Cellular Society MSC criteria [22], and which were not senescent, were frozen in FBS with 10% dimethyl sulfoxide (DMSO, Euroclone, Pero, Mi, Italy) or in Physiological solution containing 5% of human albumin and 10% DMSO. The cells were then thawed at the moment of the experiments in this study.

BM-MSCs used for this study, were analyzed for viability, immunophenotype, differential and proliferative potential to verify that the freezing had not altered the MSCs' characteristics as described in details in a previous work [18]. The BM-MSCs isolated and expanded in FBS or in iHPL were denominated, as described above, respectively FBS-MSCs and iHPL-MSCs.

2.2. Preparation of human Peripheral Blood Mononuclear Cells (PBMC)

PBMC were separated from buffy coats by centrifugation on a Ficoll Hystopaque density gradient. The buffy coats were obtained from the Blood Component Production and Validation Center, City of Health and Science of Turin, S. Anna Hospital, from healthy donors, after informed consent, using an automated blood component separator (Compomat G5, Fresenius Kabi; Bad Homburg, Germany). Donors were negative for infectious markers (Hepatitis B and C, HIV 1-2 and Treponema pallidum) in accordance with Italian laws and European guidelines.

2.3. Co-culture MSC/T cells

All co-culture experiments were performed following the same experimental design previously described [18]. Briefly, BM-MSCs were plated in 6, 24 or 96 well plates or T-flasks ($25cm^2$) containing total PBMC from an unrelated donor (the MSCs/T cell ratio was 1:10). To trigger T lymphocytes, PBMC were stimulated with Phytohemagglutinin (PHA) (2.5 μg/mL). We also isolated T naïve cells through magnetic separation with CD45RA microbead and used a cocktail of antibodies and cytokine to trigger lymphocytes in Th1 and Th2 subsets as described in [18].

The culture conditions were: 1) MSCs alone; 2) Unstimulated PBMC; 3) PHA-stimulated PBMCs (PHA-PBMC); 4) Co-culture of MSC-T cells with unstimulated PBMCs; 5) co-culture of MSCs with PHA-PBMC; 6) Th1 or Th2 lymphocytes; 7) co-culture of MSCs with stimulated Th1 or Th2 induced CD45RA+.

The supernatants were collected to analyze Th1,Th2 and Th17 cytokine release analysis (IL-2, IL-12, INF-γ and TNF-α and IL-10, IL-4, IL-6, IL-17 respectively) performed by ELISA (Mabtech, ELISA Assay).

For proliferation test, MSCs were irradiated at 3000 rad and plated into 96-well plates in 100 μL of complete α-MEM medium ($2×10^3$ cells/well) in triplicates. After 24 hours, $2×10^4$ PBMC were added to wells (in 100 μL volume) containing or lacking MSCs in the presence of the mitogen PHA (2.5 μg/mL). The MSC/PBMC ratio was 1:10. The condition without PHA was used as controls. Four hours before the end of the 72-hour co-culture ^{3}H-thymidine (1 μCi (0.037 MBq) was added. Incorporated radioactivity was evaluated after cells harvesting in counts per minute (cpm) using a 1450 Microbeta TriLux apparatus (Perkin Elmer, Boston, MA).

2.4. Cytofluorimentric analysis

PBMC characterization was described in details in a previous work [18]. All antibodies (mAb) were from Becton Dickinson Antibodies and were used in the following panels with the appropriate amount of antibody and time of incubation (20 minutes), according to the antibody titration as described by Rustichelli and colleagues [23]: anti-human CD45RA-FITC /CD45RO-PE/CD3-peridinin-chlorophyll protein cyanine 5.5 (PerCP-Cy5.5)/CD8-APC, CD45RA-FITC/CD45RO-PE/CD3-PercP-Cy5.5/CD4-APC. The labeled cells were thoroughly washed with PBS 1X (200 g for 10 minutes) and analyzed on a FACSCanto II (Becton Dickinson) with the DIVA software program.

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The percentage of positive cells, calculated using the unstained cells as a negative control, was used to calculate the absolute number on the basis of the cell number counted after 5 days of co-culture.

2.5. Evaluation of cytokine release by ELISA

The supernatants were collected for Th1 and Th2 cytokine release analysis (IL-2, IL-12, IFN-γ and TNF-α and IL-10, IL-17, IL-4, IL-6, respectively), performed by ELISA kit coated at home (Mabtech, ELISA Assay) as previously described [18]

2.6. Statistical analysis

All the data obtained in this work were analyzed by Graph PAD Prism (version 8) statistical software. In all graphics mean values with standard error f mean (SEM) were reported. The effects of FBS-MSCs and iHPL-MSCs on T lymphocytes were analyzed in the different experiments with repeated measures by two-way ANOVA. Multiple comparison Tukey test was used to compare every mean with every other mean and Dunnett test was used to compare the co-culture conditions with the control condition (PHA-PBMC).

All statistical tests were considered significant for a $P<0.05$, highly significant for $p<0.001$ and very highly significant for $P<0.0001$.

3. Results

3.1. MSC characteristics

Thawed FBS-MSCs and iHPL-MSCs, grew and reached confluence within a few days. Prior to use, the cells were analysed for immunophenotype and multipotent characteristics as demonstrated in [21]. MSCs, independently from the culture condition were negative for CD45, CD34 and CD14 and HLA-DR and were positive (over 95%) for CD90 (a membrane glycoprotein, also called Thy-1), CD105 (endoglin) and CD73. CD146 (cell surface glycoprotein MUC18) was also tested and was positive in all the samples. No statistical differences were observed between the two groups in terms of both positive cell percentages and fluorescence means of the positive markers as shown in the

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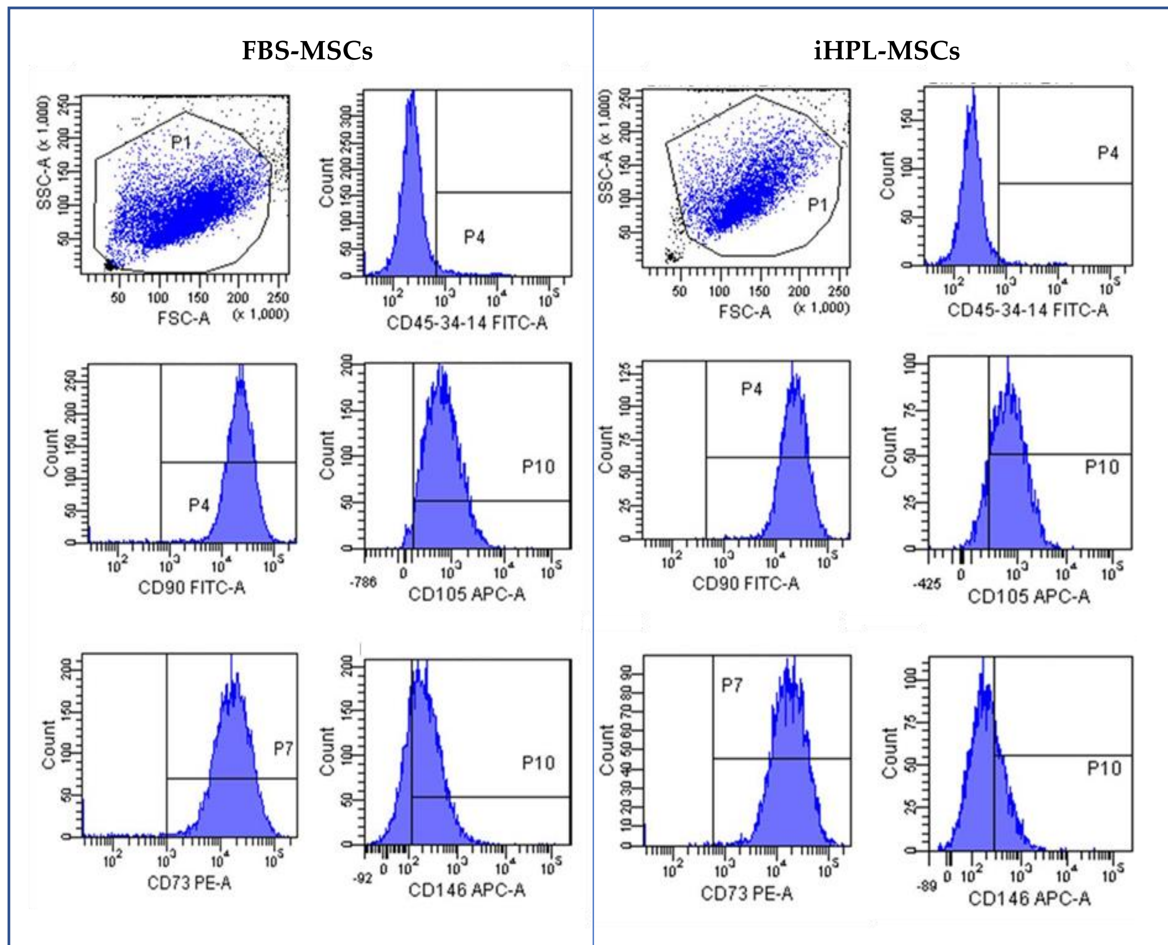

Figure 1 - Immunophenotype of representative FBS-MSCs and iHPL-MSCs cultures post thawing.

3.2 MSC/T cell interaction and Proliferative assay

The 3H-thymidine incorporation data of each experiment was expressed as a mean of counts per minute (cpm) of the triplicate and graphed in the Figure 2 with their SEM. PHA-PMSC showed high value of cpm (mean with SEM: 140265± 34868) that significantly decreased in the co-culture conditions, respectively of 33911 ± 6919 in FBS-MSCs and of 43723 ± 9339 in iHPL-MSCs. Two Anova test and multiple comparison analysis showed a significant difference between PHA-PBMC vs. PHA-PBMC + FBS-MSCs (p=0.007) and between PHA-PBMC vs. PHA-PBMC + iHPL-MSC (p=0.0016) ; no significant differences were observed between PHA-PBMC + FBS-MSC vs. PHA-PBMC + iHPL-MSC.

However different modulations in proliferative activity in Th1 and Th2 induced PBMCs were observed, without significant differences between FBS-MSCs and iHPL-MSCs.

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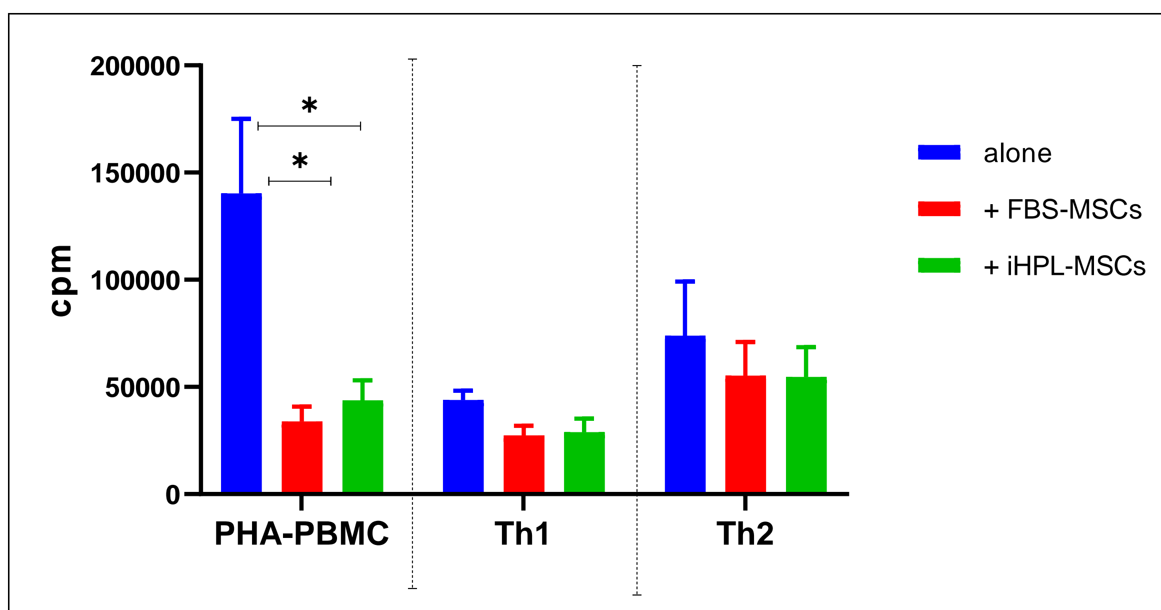

Figure 2 - Proliferative assay on stimulated PBMCs and on induced Th1 and Th2 cells alone and in co-culture with FBS-MSCs o iHPL-MSCs. Each histogram represented the mean values ± Standar Error Mean of each condition (n=5) and the symbol * indicate statistically significant difference with $p<0.05$.

3.3. T cell subsets determination

The multiparameter flow cytometric analysis allowed the identification of the following T subsets, based on the antibody combination used:

- cytotoxic T naïve cells : $CD45RA^{+}$ /$CD3^{+}$/$CD8^{+}$
- Th naïve cells: $CD45RA^{+}$/ $CD3^{+}$/$CD4^{+}$
- Cytotoxic T memory cells $CD45RO^{+}$/$CD3^{+}$/$CD8^{+}$
- Th memory cells: $CD45RO^{+}$/$CD3^{+}$/$CD4^{+}$

From the percentage obtained by cytofluorimetric analysis we calculated the absolute number of cells after 5 days of co-culture (PHA- PBMC + FBS-MSCs and PBMC + iHPL-MSCs). The data, obtained for naïve and memory T subsets from 10 experiments, were summarized in the **Figure 3**. The percentage of these subsets was strictly related to the variability of the donors. Despite we obtained a variable number in the PHA-PBMC, we observed a strong statistical significance in both the co-cultures with FBS-MSCs and iHPL-MSCs in comparison to the single culture of PHA- PBMC. Interestingly, in all the experiments the different subsets showed the same modulation trend. In particular, in PHA-PBMC, memory T cells were higher than naïve cells and, after co-culture with both FBS-MSCs and iHPL-MSCs, it was observed that: a) This ratio reversed in favour of naïve T cells, especially naïve cytotoxic subsets; b) both CD4 and CD8 memory T cell subset significantly decreased. Two Way Anova multiple comparison showed a significant increase of naïve cells CD45RA both helper (CD45RA+3+4+) and cytotoxic (CD45RA+3+8+) in co-cultures with PHA-PBMC + iHPL-MSCs respectively with $p=0.0302$ and 0.1076. The decrease of the memory cells both CD45RO+3+4 either CD45RO+3+8+ was highly significant in all co-culture conditions as shown in the **Figure 3** . In particular, comparing PHA-PBMC vs PHA-PBMC + FBS-MSCs and vs PHA-PBMC + iHPL-MSCs we observed respectively a p value=0.002 and $p=0.007$ for the CD45RO+3+4+ and a p value =0.0010 and 0.0017 for the CD45RO+3+4+.

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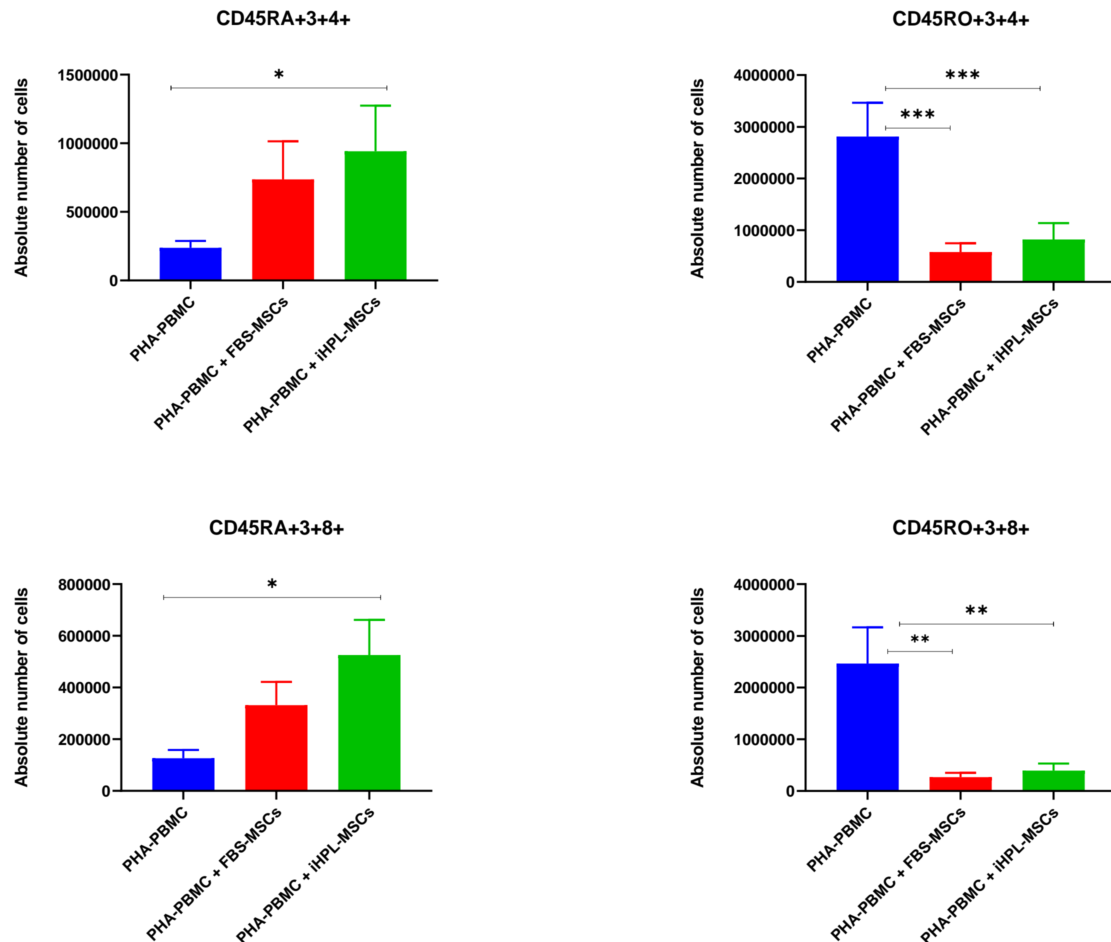

Figure 3 - T subsets evaluated in PHA-PBMC alone and in co-cultured with FBS-MSCs and iHPL-MSCs and expressed in mean values ± SEM. The symbols *, **, *** indicate statistically significant difference respectively with $p<0.05$, $p<0.01$, $p<0.001$.

3.5. Th1/Th2/Th17 cytokine release

PHA-PBMC were used as a control and the range of analysed cytokines was reported in the **Figure 4**. As also described in literature [24], PHA-PBMC showed high levels of IL-2, IL-12, TNF-α and IFN-γ and in all the co-culture conditions we observed a strong decrease in these cytokine release . We also analyse the constitutive expression of these in cytokines in FBS and iHPL-MSCs and we observed no difference between them: a)Negligible levels of Th1 cytokine, except for IL-12 which was higher than IL-12 levels in PHA-PBMC; b) Very low Th2 cytokines and 3) significant levels of IL-6 and IL-17.

For all the cytokines, the multiple comparison statistical analysis did not reveal any significant differences between FBS-MSCs and iHPL-MSCs. So we focused our statistical analyse on the effects the different MSCs on PHA-PBMC using Dunnet's test .

In the co-culture experiments we observed a significant decrease of IL-2 concentration both with FBS-MSCs (p= 0.0112) either with iHPL-MSCs (0.0007). Also IL-12 produced in high levels in PHA-PBMC decrease, even if FBS and iHPL-MSCs constitutively produced higher levels of this cytokine than PHA-PBMCs, we observed a reduction of IL-12 in the two co-culture conditions (Figure 4).

The high level of TNF-α produced by PHA-PBMC decreased in all MSC co-culture conditions as shown in Figure 4 C. Also, IFN-γ decreased in the co-cultures with MSCs (Figure 4).

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PHA- PBMCs produced moderate amounts of Th2 cytokine levels (IL-4 and IL-10), which increased in the presence of MSCs in all co-culture conditions, but without statistically significant differences in the two co-culture conditions (Figure 4).

Moreover, PHA stimulated PBMCs showed a moderate production of IL-17 and IL-6 which increased in all co-culture experiments with MSCs. The data are reported in the **Figure 4** and multiple comparisons test showed a significant increase of IL-6 respectively in the co-culture with FBS-MSCs and iHPL-MSCs (p=0.0402 and p=0.0469), but no statistically difference were observed in IL-17 level. Also here, no differences were observed between FBS and iHPL-MSCs.

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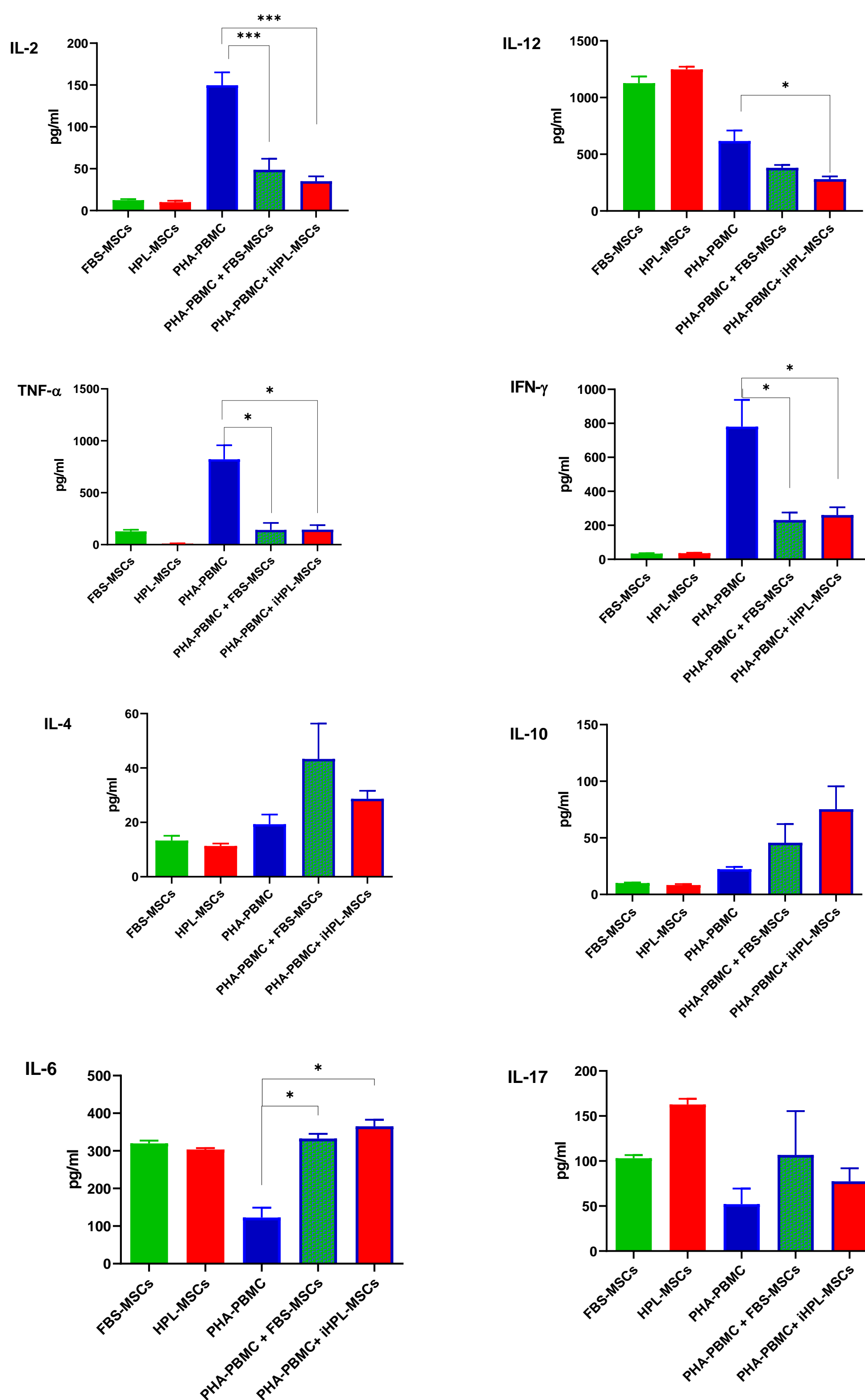

Figure 4 - Th1 (IL-2, IL-12, TNF-α.INF-γ), Th2 (IL-4, IL-10) and Th17 (IL-6, IL-17) cytokines release (pg/ml) analysed by ELISA assay in FBS-MSCs , HPL-MSCs and PHA-PBMC alone and in the co-cultures PHA-PBMC with FBS-MSCs o iHPL-MSCs in mean values ± SEM (n=3). The symbol *, *** indicate statistically significant difference with $p<0.05$, $p<0.001$.

4. Discussion

Regenerative medicine is of growing interest in biomedical research and in this context, MSCs are a promising tool for cell therapies for their multipotent, bystander and immunomodulant proprieties. For these reasons, MSCs are used for a very wide range of therapeutic applications, the majority of which are in Phase I, Phase II, or a mixture of PhaseI/II studies. Some phase III and IV are also in progress (www.clinicaltrial.gov). A variety of protocols are described for the GMP MSC production, some of these also using selected FBS, other xeno-free components or HPL or plasma. Since MSCs are considered ATMP, qualified protocols with standard and precise characteristics, large-scale quality, and relatively low-cost production need to be developed using xeno-free media for clinical-grade expansion [25,25,26]. In the last years we have been setting up the methods to isolate and expand MSCs for clinical use and we, also, demonstrated that iHPL, prepared in house from a big pool of donor platelets underwent to pathogen inactivation by psoralen, was more efficient and safer than FBS to isolate BM-MSCs in GMP condition [21,27]. As emerged from the analyses of the pluripotency markers, such as Oct-3/4 and NANOG, these proliferative and differentiative properties of the iHPL-MSCs might be linked to more immature stemness in comparison with FBS-MSC. These observations indicate that iHPL-MSCs contain a subpopulation of multipotent stem cells, which might be the precursors of MSCs, with a more primitive phenotype than those of FBS-MSCs [8].

In this study, we tested if MSCs isolated and expanded in iHPL preserve their immunomodulant proprieties analysing their effects on T lymphocytes in comparison with MSCs isolated and expanded in FBS whose immunomodulating properties have already been described in the literature [17,18,28].

The ability to modulate the alloreactive immune response has been documented for MSCs derived from human BM; concurrently comparative studies between FBS-MSCs and HPL-MSCs were already performed and indicate that HPL, used as supplement for MSCs media, supports immune modulation at least to the same extent than FBS in addition to its role during MSC isolation and expansion [29–31]. No comparative studies of immunomodulation were performed between FBS-MSC and MSC cultured in iHPL, which is even safer and more GMP compliant than HPL for MSC expansion [21].

BM-MSCs cultured in alpha-MEM + 10% of FBS or 10% of iHPL (FBS-MSC and iHPL-MSC) aliquots were thawed and: i) immunophenotipic (Figure 1), proliferative and differentiative characteristics were not modified (data not shown) by cryopreservation, and ii) their immunomodulant effects on T cells of healthy donors were compared. The results, in accordance with literature, showed that MSCs cultured both in FBS and iHPL are multipotent stem cells with the immunophenotyic characteristics and differentiative potential as established by guidelines by the Cellular Therapy Society [32].

Since T-cells are the primary cells in adoptive immune response, an evaluation and comparison of the inhibitory effects of MSCs on total activated T-cells with a potent mitogen (PHA) and on naïve T-cells induced to differentiate in Th1 and Th2 effector cells. A T-cell proliferation assay showed inhibitory effects on PHA- PMBC in all co-culture conditions, while the proliferation data obtained in induced Th1 and Th2 effector cells were not homogenous and statistically significant. However, these results suggest that T cell inhibition might be strictly related to an interaction of these cells with other cells from innate immunity (such as dendritic cells and NK) not present in co-cultures with Th1- and Th2- induced cells alone.

No significant differences were found in the inhibition of T cell proliferation in co-culture with FBS-MSCs or iHPL-MSCs. When we analysed the different T subsets, we observed a statistically significant increase of naïve T cells in the co-cultures with iHPL-MSCs as well as a strong decrease of memory T cells in both the co-cultures with FBS-MSCs and iHPL-MSCs. Indeed, the presence of MSCs induced a reversal of the ratio (compared to stimulated PHA-PBMC) of these subsets in the co-cultures in favour of both CD4+ and CD8+ naïve T cell subpopulations. iHPL showed a higher effects of naïve T cells than FBS in the co-culture with MSCs.

An important mechanism by which MSCs have beneficial effects in cell therapy is the paracrine action of secreted cytokines. Major cytokines associated with pro-inflammatory and anti-

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inflammatory functions were analysed and compared. A noteworthy finding was a distinctly high concentration of IL-12 in MSCs. Independently from the culturing conditions, MSCs also produced a moderate concentration of TNF-α and negligible amounts of IL-2 and IFN-γ. PHA-stimulated PBMC showed high levels of Th1 cytokine as reported in Lee et al [33]. In all co-culture experiments, we observed a decrease of all Th1 cytokines. The interaction MSCs/T cells might block the Th1 polarization because this phenomenon was also found for IL-12 produced in high concentrations also by MSCs. It is interesting to note that IL-4 and IL-10, the major anti-inflammatory cytokines, increased in co-cultures with FBS-MSCs and iHPL-MSCs but without reporting statistical differences .

The same effect was observed for the Th17 cytokines. Moreover, the MSCs, independently from the culturing condition, produced high levels of both IL-6 and IL-17, so it is difficult to understand the significant increase of these cytokines in the co-culture with PHA-activated PBMCs.

As showed in literature also here, MSCs in the two different experimental conditions inhibited or limited inflammatory responses and promote the mitigating and anti-inflammatory pathway especially on Th1 cells, leading to a paradoxical increase of pro-inflammatory Th17 cells. A mechanism that could explain the late stimulating effect of MSCs on pro-inflammatory Th17 cells is the up-regulation of IL-6 levels in the cultures, since IL-6 is a main mediator of Th17 cell differentiation [28,34,35]. Furthermore, in agreement with the results in this study, IL-6 might also inhibit the differentiation of Th1 subset. IL-4 and IL-10 are indicative of a Th2-deviated immune response and might be produced by a cellular compartment different from the T cells in PBMC. However, from these studies, conclusions cannot be drawn on the separate roles of the different cytokines in either mediating inhibition directly or inducible inhibition because of the complex interaction of many factors.

On the base of our results we observed that iHPL preserve all analysed immunomodulant proprieties of MSCs as well as FBS and for some effects it is also more efficient. For this reason, they may be more effective than FBS-MSCs in the control of immune-mediated pathologies, particularly GVHD. On the other hand HPL-MSCs appeared particularly useful in regenerative medicine [16].

The advantage of HPL in obtaining MSCs in vitro expansion is related not only to the increment of proliferation but also to the fact that, different from FBS, HPL does not induce antibody responses in patients [38,39] and its inactivation with psoralen makes it even safer than HPL blocking and inactivating the replication of viruses, bacteria and leukocytes in PLT concentrates [11,38]. The fact that we and other [11] found differences in immunomodulant properties of FBS-MSsC and HPL/iHPL-MSCs that, in our case, are not are not significant, offer an interesting clue regarding possible functional differences in MSC output and clinical applications [21].

In Conclusion, for each experiment a contingency table is given (Table 1- Summary of results) where the MSC proprieties and their effects on T-cells are considered as strong, higher or moderate and it can thus be stated that:

- iHPL show a greater proliferative, differentiative and stemness potential than FBS-MSC [8]
- Both FBS-MSCs and iHPL-MSCs showed a potent immunomodulant effect on T-cells without strong significant differences between them.
- This study confirm that iHPL, used as medium supplement, may be considered a more efficient additive alternative to FBS for a GMP-compliant MSC expansion.

The development of new strategies for the large scale production of these cells, according to current regulations, including GMP, represents a fundamental step to allow their use in effective therapeutic approaches..

The use of iHPL as an alternative to FBS to isolate and expand MSC confirmed that it is possible to obtain a number of MSCs in large scale for clinical doses which maintained intact all their characteristics, including their immunomodulant properties. The application of iHPL as medium supplement, further reduce manufacturing time, limiting the passages and reducing the starting volume of BM. Moreover, the pathogen inactivation treatment did not modify the characteristics of HPL, made it safer and more suitable for MSC isolation and expansion for clinical use and might be a requirement for GMP MSC expansion.

This study might well open the way for new ambitious projects which will allow the identification of new and more advantageous way to culture MSCs, which was safer than those

commonly used, in order to create a bio-bank of ready to use MSCs for clinical use. The possibility of banking MSCs isolated from BM or other sources under GMP conditions in an AIFA (Agenzia Italiana del Farmaco – the Italian Medicine Agency) accredited Cell Factory might represent a new scenario for their clinical use in cell therapy protocols providing a continuous supply of cells to treat patients with acute GVHD after allogeneic hematopoietic stem-cell transplantation, solid organ transplantation or in inflammatory and autoimmune diseases.

5. Conclusions

Table 1 summarized the results obtained in all experiments of this study. The symbols "+++", "++" and "+" or "-/+" indicate the expression or effect grade as very strong, high and moderate, statistically significant, respectively.

Table 1. Summary of results (* indicate that data are statistically significant).

	FBS - MSC	iHPL- MSC
MSC characteristics		
MSC immunophenotype*	+++	+++
Proliferative potential*	+	+++
Pluripotency marker expression (protein)	+	+++
MSC Th1 cytokine production	++	++
MSC Th2 cytokine production	-/+	-/+
MSC Th17 cytokine production	++	++
Effect on T-cells		
Inhibition T proliferation*	++	++
Naïve T helper Increase*	-/+	+
Naïve T cytotoxic Increase*	-/+	+
Memory T helper Decrease*	+++	+++
Memory T cytoxic Decrease*	++	++
Th1 cytokine decrease*	++	+++
Th2 cytokine release increase	+	+
Th17 cytokine release increase*	++	++

Author Contributions:

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The authors declare no conflict of interest.

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