SINGLE-CELL DERIVED CLONES FROM HUMAN ADIPOSE STEM CELLS PRESENT DIFFERENT IMMUNOMODULATORY PROPERTIES

Short title: ***Immunomodulation by adipose mesenchymal cell clones***

José M Sempere[1], Pascual Martinez-Peinado[1], María I Arribas[2], Juan A Reig[2], Mari L De La Sen[1], José J Zubcoff[3], Mario F Fraga[4,5], Agustin F Fernández[5], Alfredo Santana[6] and Enrique Roche[2,7]

(1) Immunology Division, Biotechnology Department, University of Alicante (Spain).

(2) Biochemistry and Cell Therapy Unit, Institute of Bioengineering, University Miguel Hernandez, Elche (Spain).

(3) Department of Statistics, University of Alicante (Spain).

(4) Department of Immunology and Oncology, National Center for Biotechnology, CNB-CSIC, Cantoblanco, Madrid (Spain).

(5) Cancer Epigenetics Laboratory, Institute of Oncology of Asturias (IUOPA), HUCA, University of Oviedo, Oviedo (Spain).

(6) Research Unit, Gran Canaria Hospital Dr Negrin and Genetic Unit. Childhood Hospital Complex, Las Palmas, Canary Islands (Spain).

(7) CIBERobn (Fisiopatología de la Obesidad y la Nutrición CB12/03/30038) Instituto de Salud Carlos III, Spain.

Corresponding author:

Enrique Roche.

Instituto de Bioingeniería, Universidad Miguel Hernández.

Avda de la Universidad. 03202-Elche, SPAIN

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Phone: 34965222029, Fax: 34965222033, eroche@umh.es

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Abbreviations used:

GEO, Gene Expression Omnibus; GVHD, graft-versus-host-disease; hASCs, human adipose stem cells; ISCT, International Society for Cellular Therapy; MFI, median fluorescence intensity; MSCs, Mesenchymal stem cells; PBMC, Peripheral blood mononuclear cells.

SUMMARY

Human adipose mesenchymal stem cells are a heterogeneous population, where cell cultures derived from single cell-expanded clones present varying degrees of differential plasticity. This work focuses on the immunomodulatory/anti-inflammatory properties of these cells. To this end, 5 single cell clones were isolated (generally called 1.X and 3.X) from 2 volunteers. Regarding the expression level of the lineage-characteristic surface antigens, clones 1.10 and 1.22 expressed the lowest amounts, while clones 3.10 and 3.5 expressed more CD105 than the rest and clone 1.7 expressed higher amounts of CD73 and CD44. Regarding cytokine secretion, all clones were capable of spontaneously releasing high levels of IL-6 and low to moderate levels of IL-8. These differences can be explained in part by the distinct methylation profile exhibited by the clones. Furthermore and after lipopolysaccharide stimulation, clone 3.X produced the highest amounts of pro-inflammatory cytokines such as IL-1β, while clones 1.10 and 1.22 highly expressed IL-4 and IL-5. In co-culture experiments, clones 1.X are altogether more potent inhibitors than clones 3.X for proliferation of total, $CD3^+T$, $CD4^+T$ and $CD8^+T$ lymphocytes and NK cells. The results of this work indicates that adipose stem cell population is heterogeneous in cytokine production profile, and that isolation, characterization and selection of the appropriate cell clone is a more exact method for the possible treatment of different patients or pathologies.

INTRODUCTION

Mesenchymal stem cells (MSCs) are a population of stem cells that can be isolated from a variety of tissues, including bone marrow [1], umbilical cord [2], foetal tissues [3], amniotic liquid [4], synovial membrane [5], dental pulp [6] and adipose tissue [7]. Although MSCs are found in very small quantities *in vivo*, they can be easily expanded *in vitro* and represent an attractive therapeutic tool for regenerative medicine. In fact, MSCs are multipotent and, as such, can give rise to a variety of mesodermal phenotypes, including osteogenic, adipogenic, chondrogenic, muscle, or stromal cells [8-15]. In addition, MSCs possess unique immunomodulatory properties, being capable of suppressing T cell responses and modifying dendritic cell differentiation, maturation, and function. Furthermore, these cells are not inherently immunogenic, failing to induce alloreactivity to T cells and freshly isolated natural killer (NK) cells [16], making them an attractive tool in cell therapy protocols for the treatment of inflammatory-related diseases.

The immunomodulatory properties exhibited by MSCs come in part from the expression of specific protein markers. Unfortunately, as of yet there is no one specific marker that identifies MSCs, thus to identify these cells several surface markers are used. In this respect, one attempt to standardize the phenotypic characterization of MSCs came for the International Society for Cellular Therapy (ISCT). The ISCT proposed that MSC populations must be positive at least for the following surface markers: CD44, CD73, CD90 and CD105 [17-21]. Additionally, these cells should lack the expression of haematopoietic antigens such as CD34, CD45 as well as markers for monocytes, macrophages and B cells [21]. CD44 is an adhesion molecule involved in a wide variety of cellular functions including lymphocyte activation, recirculation and homing [22]. CD73 catalyses the conversion of purine 5'-mononucleotides to nucleosides, mainly AMP. Its expression in Treg cells seems to be a part of their regulatory mechanisms [23]. CD90 antigen is known to act to some extent as a CD28-substitute

activating signal for T-cell receptor signalling [24]. CD105 (endoglin) is part of the TGF-β1 receptor complex. Also, TGF-β signalling [25] is involved in the cytoskeletal organization, affecting cell morphology and migration [26].

The majority of the studies carried out to phenotypically characterize MSCs have been performed using MSCs cultures without considering the fact that they are a heterogeneous population of cells, as previously shown [27]. In an attempt to better characterize MSCs, in this paper we have analysed for the first time the expression of some of the aforementioned surface antigens in different clones of human MSCs isolated from the adipose tissue (hASCs), while at the same time have identified their Th1/Th2 cytokine profile as well as their ability to inhibit lymphocyte proliferation in culture. Part of the differences observed between clones could be explained by the different pattern of DNA methylation. Finally, potential differences were analysed in the clones that may be applied in a near future in various cell therapy protocols.

MATERIALS AND METHODS

Cells and reagents

This study was conducted according to guidelines written in the Declaration of Helsinki and all procedures involving human subjects/patients were approved by the Ethical Committee of University Miguel Hernandez. Written informed consent was obtained from the two subjects. Five different hASCs clones isolated from the two healthy subjects were used for all of the experiments (clones 1.10, 1.22, 1.7, 3.10 and 3.5). Clones 1.10, 1.22 and 1.7 were obtained from one of the subjects and will be referred altogether as clones 1.X for further analysis. Clones obtained from the second subject (3.10 and 3.5) will be referred altogether as clones 3.X. Clones were isolated and cultured as previously described [27]. Briefly, processed lipoaspirates were plated at limiting confluence to isolate single cells. Cultures were incubated in cloning medium (HAM F12 supplemented with 20% FCS, 100 U/mL penicillin, 100

μg/mL streptomycin and 15 mM HEPES) until the formation of well-defined colonies (30-50 cells). Derived colonies were harvested using sterile cloning rings and expanded in cloning medium until the cultures reached 70-80% of confluence in a P100 dish. At that time, culture medium was changed by control medium (DMEM supplemented with 10% FCS and antibiotics) and cells were expanded for freezing or cultured in order to carry out the experiments. The mean dubbing time of clones ranges between 60-72 h.

Cells cultured for 12-15 passages were used for this study. Peripheral blood mononuclear cells (PBMC) were also obtained from healthy subjects. Antibodies against surface membrane antigens of ASCs and PBMC were obtained from BD Bioscience (Becton-Dickinson, Franklin Lakes, NJ, USA) and eBioscience (San Diego, CA, USA). Flowcytomix^{TM} Multiplex Test including recombinant cytokines and monoclonal antibodies against cytokines was obtained from eBioscience, Trypsin from Life Technologies Corporation (Carlsbad, CA, USA) and carboxyfluorescein succinimidyl ester (CFSE) from Sigma-Aldrich (St Louis, MO, USA), mitogens, antibiotics (penicillin/streptomycin), fungizone, foetal calf serum (FCS) and culture media from Sigma Aldrich and Life Technologies Corporation. FACSCANTO and FACSCALIBUR (BD Bioscience) were used for flow cytometry.

hASC-membrane phenotype characterization

hASC-clone cultures were first washed twice in phosphate buffered saline (PBS), then trypsinised and resuspended in mesenchymal culture medium (DMEM 25 mM glucose, 10% FCS, 1% penicillin/streptomycin), centrifuged, incubated in the dark for 30 min at 4ºC with a mix of fluorochrome-conjugated monoclonal antibodies against different surface antigens (CD34-FITC, CD44-PE-Cy7, CD45-FITC, CD73-PE, CD90-PerCP-Cy5 and CD105-APC, eBioscience, Becton Dickinson) and washed again to eliminate the antibody excess. Analysis of the membrane phenotype from the different clones was carried out by direct immunofluorescence and flow cytometry (FACSCANTO, BD Bioscience) (n=3).

Analysis of ASC-Th1/Th2 cytokine profile

hASC clones were first washed twice in PBS, then trypsinised (trypsin + 0.05% EDTA for 5 min at 37ºC and 5% CO_2) and finally resuspended in mesenchymal culture medium (DMEM 25 mM glucose, 10% FCS, 1% penicillin/streptomycin), seeded ($2x10^4$ cells per well) into flat-bottom 96-well plates and incubated for 96 hours at 37ºC and 5% CO_2. Cells were stimulated with lipopolysaccharide (LPS) (1 or 0.2 μg/ml) or phorbol-myristate acetate (PMA) (50 ng/mL) + ionomycin (1 μM), all from Sigma-Aldrich, either immediately after trypsinisation or 4 days later. Non-stimulated cells were used as control. Spontaneous and post-stimulation secretion of Th1/Th2 cytokines in supernatants were measured by Flow Cytometry (FACSCALIBUR, BD Bioscience) through the Flowcytomix^{TM} Multiplex Test (eBioscience) (n=3). The following cytokines were analysed for each clone: IL-1β, TNF-α, TNF-β, IL-2, IL-4, IL-5, IL6, IL-8, IL-10, IL-12 and INF-γ. A whole kinetics was performed for each cytokine at 2, 4, 8, 12, 24, 48, 72 and 96 h of culture.

Determination of DNA methylation profile

Microarray-based DNA methylation profiling was performed on all samples with Infinium HumanMethylation27 BeadChip arrays (Illumina, San Diego, CA) as previously described [28]. It accomplishes this high multiplexing by combining bisulfite conversion of genomic DNA and whole genome amplification sample preparation with direct, array based capture and enzymatic scoring of the CpG loci. The assays interrogate the chemically different loci using two site-specific probes, one designed for the methylated locus (M bead type) and another for the unmethylated locus (U bead type). The methylation level for the interrogated locus is determined by calculating the ratio of the fluorescent signals from the methylated versus unmethylated sites. The ratio of fluorescent signals is then computed from the two alleles according to the following formula: Beta = Max(M,0)/[Max(U,0) + Max(M,0) + 100]. The beta value is a quantitative measure of DNA methylation levels of specific cytosine-

guanine dinucleotide (CpG), and ranges from 0 (completely unmethylated) to 1 (completely methylated). Every beta value in the array platforms is accompanied by a detection p-value. We based the filtering criteria on these p-values reported by the assays. All probes with detection p-values >0.01 were removed. The clustering heatmaps using methylation data were performed with BeadStudio software (Illumina) and Microsoft Excel tools.

The microarray data from this study have been submitted to the NCBI Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo) under accession number GSE44222.

In order to study coincidences between the methylation pattern and the gene expression profile, RT-PCR assays were carried out on specific genes by using the following primers (Fw: forward primer, Rv: reverse primer). The product size (bp), annealing temperature/cycles and accession number were indicated:

- IL-4: Fw 5'-CTGCTAGCATGTGCCGGCAA-3',
  Rv 5'-GACAGGAATTCAAGCCCGCCA-3', 331 bp, 60ºC/35, NM_000589.
- IL-6: Fw 5'-CTCCTTCTCCACAAGCGCCTT-3',
  Rv 5'-GGCAAGTCTCCTCATTGAATC-3', 329 bp, 60ºC/35, NM_000600.
- IL-8: Fw 5'-TCTGCAGCTCTGTGTGAAGGTG-3',
  Rv 5'-GCCCTCTTCAAAAACTTCTCCAC-3', 242 bp, 60ºC/35, NM_000584.
- IL-10: Fw 5'-CTTTAAGGGTTACCTGGGTTG-3',
  Rv 5'-GAAATCGATGACAGCGCCGTA-3', 170 bp, 59ºC/36, NM_000572.
- TNF-α: Fw 5'-CCAGAGGGAAGAGTTCCCCA-3',
  Rv 5'-CCCTCAGCTTGAGGGTTTGCT-3', 126 bp, 60ºC/35, NM_000594.

The effect of methylation on gene expression was assessed by incubating clone 1.10 in the presence of the demethylating agent 5-aza-2'-deoxycytidine (Sigma). To this end, 5 x 10^4 cells/well were seeded in a 24 well-plate and incubated for 24 h. Next day, 5-aza-2'-deoxycytidine was added at different concentrations ranging from 5 μM-1 mM and cells were

incubated 24 additional hours. Trypan blue exclusion test was performed at the end of incubation to determine viability. A concentration of 20 μM 5-aza-2'-deoxycytidine (80% viable cells) was selected for subsequent gene expression experiments.

hASC-PBMC co-cultures

The studies were performed by co-culturing all of the hASC clones with PBMC isolated from 7 unrelated donors by gradient density centrifugation with Ficoll-Hypaque from GE Healthcare (Chalfont, St Giles, UK). PBMC were washed twice in culture medium (RPMI supplemented with 10% FCS, 1% antibiotic+fungizone, 1% glutamine). hASC clones were first washed twice in PBS, then trypsinised as previously mentioned and resuspended in the same RPMI medium that was also used to resuspend PBMC. Cells were finally plated in triplicates onto flat-bottom 96-well plates in a final volume of 250 μl/well, containing hASC/PBMC at the rate of 1:10 (10.000/100.000) or 1:5 (20.000/100.000). Prior to be cultured PBMC were primed with CFSE (Sigma-Aldrich) for 5 min according to Parish [29].

Proliferation assay

To analyse the effect that hASC clones may have on lymphocyte proliferation, PBMC alone or co-cultured with hASC (n=7) were stimulated with phytohemagglutinin (PHA) (10 μg/ml) from Sigma-Aldrich or anti-CD3/anti-CD28 from Becton-Dickinson and further incubated for 5 days at 37ºC and 5% CO_2. Unstimulated cells for both conditions were used as their respective negative controls.

After the 5-day culture, PBMC were labelled with a combination of anti-CD3, CD4 and CD8 monoclonal antibodies and proliferation was further measured by flow cytometry (FACSCANTO, BD Bioscience) on total lymphocytes as well as on $CD3^+/CD4^+$, $CD3^+/CD8^+$ and $CD3^-/CD8^+$ lymphocyte subsets.

Statistical analysis

Statistical analysis was performed with the free *R* software. Data are presented as the mean± the standard error of the mean (SEM) from 3 or more experiments. Analysis of variances (ANOVA) was used to test the type and levels of secreted cytokines for the different clones according to the time of culture, as well as the effect of clones on lymphocyte proliferation. When the ANOVA F-test found significant differences between levels, post-hoc analyses were conducted using the most restrictive version of Tukey HSD (Honest Significantly Differences) test with the p-value adjusted by the Bonferroni method.

Cytokines were divided into two models since their behaviour throughout time were clearly different. One model was performed to analyse IL-6 and IL-8 cytokines, while the other model contained the rest of cytokines. Each model included clone and cytokine as the fixed factor and time as the random factor. Regarding lymphocyte proliferation, a descriptive analysis was first performed to obtain information concerning the proliferation rate under the different culture conditions. Then, the ANOVA test was used to analyse the data by using two factors (lymphocyte subsets and clones). When no interactions were detected, only one factor was considered to focus on differences between clones. To study the impact of the patient factor on clone behaviour we also analysed the differences between clones 1.X and 3.X.

RESULTS

Membrane phenotype of hASC

Among the clones studied, none expressed CD34 (Figure 2B) or CD45 (not shown), while 85-98% of the cells were positive for CD44, CD73, CD90 and CD105 (Figure 1). Although they were not significantly different in terms of membrane antigen expression, some specific differences were detected among the clones, especially with regard to the percentage of expression of CD90 and CD105 (higher percentage in clones 3.X). Regarding the percentage of cell clones coexpressing the four positive antigens CD73, CD90, CD44 and CD105, three different clone phenotypes can be distinguished (Figure 2A); the majority of clones 3.X (more

than 90%) coexpress these markers, while in clones 1.10 and 1.22 coexpression was only detected in 70%, and 80% in clone 1.7. With regard to median fluorescence intensity (MFI) values, clones 1.10 and 1.22 seemed to express lower amounts of antigens than clones 1.7, 3.5 and 3.10, with clone 1.22 expressing the lowest amounts of all the antigens studied (Figure 2B). CD90 was the antigen with the lowest levels of expression, being similar in all the clones analysed (Figure 2B). Clone 3.5 presented the highest levels of expression of CD105, while clone 1.7 presented more CD73 and CD44 than the rest of the clones (Figure 2B).

Analysis of Th1/Th2 cytokine profile in hASC clones

Basal secretion or in response against different stimuli

Similar expression levels of IL-6 and IL-8 were detected in all the clones both under basal culture medium (see Figures 3A and 3B respectively) and after stimulation with LPS or PMA+Ionomycin (no significant differences). Stimulation immediately after tripsinisation of hASCs or 4 days later did not exert any influence on the cytokine levels (data not shown).

With regard to the rest of the cytokines analysed, they were only expressed after stimulation. Although the cytokines secreted after stimulation of hASCs with LPS or PMA+Ionomycin were almost the same in all of the clones, LPS seemed to be the most powerful stimulus, being 0.2 μg/ml the optimal concentration to obtain the highest cytokine levels (Figure 3C). As observed for IL-6 and IL-8, no differences were detected in the type or amount of secreted cytokines according to the moment of stimulation, i.e. after trypsinisation or 4 days later (data not shown). No secretion of IL-10 or IL-12 was detected in any of the hASCs clones.

Kinetics of secreted IL-6 and IL-8

IL-6

The 5 clones produced high amounts of IL-6 (Figure 3A). Clones 3.10 and 1.22 presented faster kinetics, reaching high levels of this cytokine in the first 2-4 h of culture. The kinetics of clone 3.5 was slightly slower, producing the most amounts after 24-72 h. Clones 1.7 and

3.10 reached their higher levels at the end of the crop and continued increasing after 96 h. Finally, clone 1.22 presented the lowest levels of IL-6 after 96 h of culture.

IL-8

The 5 clones produced mild to moderate levels of IL-8 (Figure 3B). Two groups can be distinguished according to their kinetics. The first group (clones 1.10 and 3.5) presented slower kinetics. The second group (clones 1.22, 1.7 and 3.10) presented faster kinetics, reaching levels above 1000 pg/ml in the first 2-4 h of culture. Clone 1.7 secreted the highest amounts of IL-8, while clone 1.22 secreted the lowest. Clones 1.10, 3.5 and 3.10 presented a tendency to increase production after 96 h of culture.

Levels of other secreted cytokines after LPS stimulation

IL-1β

Clones 3.5 and 3.10 produced the highest amounts of this cytokine, followed by clone 1.10 (Figure 3C). The first two clones peaked after 24 h of culture and clone 1.10 after 48 h (not shown). Clone 1.7 only produced slight amounts of this cytokine and had a faster kinetics, reaching a peak in 4-6 h of culture (not shown).

TNF-α

Only clones 1.22 and 1.10 produced this cytokine at very low levels (~10 pg/ml) (Figure 3C).

TNF-β

All clones produced low levels of TNF-β (Figure 3C). Clones 1.X exhibited faster kinetics than clones 3.X, reaching a peak within 12 h of culture. Clone 3.10 presented the slowest kinetics (peaks at 48 h) (not shown).

IL-2

All clones produced low to moderate amounts of IL-2 (Figure 3C). As in other cases, the kinetics of clones 1.X was the fastest, reaching its peak in the first 4-8 h of culture. Clone 3.5 peaked at 12-24 h and clone 3.10 continued increasing after 72 h (not shown).

IFN-γ

Only clone 1.22 produced this cytokine (~25 pg/ml) (Figure 3C).

IL-4

All clones produced low to moderate amounts of IL-4 (Figure 3C). Clones 1.X presented the fastest kinetics, peaking in the first 4-8 h of culture. Clones 3.X peaked about 12-24 h (not shown).

IL-5

Clones 1.X and 3.10 produced low to moderate amounts of IL-5 (Figure 3C). Clones 1.7 and 1.22 presented the fastest kinetics, peaking in the first 4-8 h of culture. Clones 1.10 and 3.10 peaked at 48 and 24 h, respectively (not shown).

Cytokine gene methylation pattern of the different clones

Altogether, the results suggest that there is heterogeneity between the different hASC clones regarding cytokine secretion and kinetics. This heterogeneity may be explained in part by the DNA promoter methylation profile displayed for the genes encoding cytokines, receptors and related molecules in the different hASCs clones at basal/unstimulated incubation conditions. In this context, genes with lower expression values coincided with those showing higher DNA methylation values (Figure 4). On the other hand, genes that are expressed in basal conditions (i.e. IL-6 and IL-8) display low methylation scores (Figure 4). In addition, a clustering heatmap using exclusively the methylation signals of 280 genes related with cytokine signalling showed that each particular clone presented a specific promoter DNA methylation signature (Figure 5), thus confirming this heterogeneity.

In order to study the role of methylation on the modulation of cytokine gene expression, clone 1.10 was incubated in the presence of the demethylating agent 5-aza-2'-deoxycytidine as indicated in Materials and Methods. Under these conditions, IL-10 and TNF-α that were not expressed in basal conditions (Figure 4), are expressed now (Figure 6). On the other hand, IL-

4 is not expressed in the absence (Figure 4) or in the presence of aza-deoxycytidine (Figure 6). These results suggest that DNA methylation might be an instrumental mechanism in controlling the expression of specific cytokines (i.e. IL-10 and TNF-α), but not for others (i.e. IL-4). In any case, these results have to be interpreted with caution because the magnitude of changes that occurred in the methylation pattern of the DNA after incubation of clone 1.10 with 20 μM aza-deoxycytidine is unknown at present.

hASC-PBMC co-cultures and proliferation assay

Proliferation was not observed in unstimulated PBMC cells alone or with hASC. PHA-based stimulation triggered proliferation in the majority of the lymphocyte subsets (data not shown). Although the PHA-induced proliferation rate was slightly different for each PBMC donor, to homogenize results, lymphocyte proliferation was expressed as part of a unit, according to the following formula: [Cell proliferation (parts per unit)= (% proliferation of PBMC+PHA with or without hASC)-(% proliferation of PBMC with or without hASC)] / [(% proliferation of PBMC+PHA)-(% proliferation of PBMC)].

All the ANOVA comparisons between the proliferation rates observed in hASC clones/PBMC co-cultures and that of lymphocytes alone (PBMC) were highly significant ($p < 0.000$) (Figures 7 and 8). The presence of hASCs in the cultures resulted in a significant decrease in proliferation in both total lymphocytes and their subsets ($p<0.0001$). The rate of inhibition, however, differed among the clones. Depending on their behaviour, two groups can be distinguished (Figure 7). Clones 1.X (grey bars) had a significantly stronger suppressive effect than clones 3.X (white bars) for total lymphocytes ($p= 0.0044$), $CD3^+$T lymphocytes ($p=0.00504$), $CD4^+$T lymphocytes ($p=0.0254$), and NK cells ($p= 0.0121$), but not significant for $CD8^+$ T lymphocytes ($p= 0.349$).

Also, the 1.X clones were more suppressive than 3.X (Figure 8). Figure 8 shows the five clones sorted in descending order according to their inhibitory effect on lymphocyte

proliferation. A descriptive individual analysis showed that when total and $CD3^+$ lymphocytes were considered, clone 1.22 was the most inhibiting, while clone 3.10 was the least. However, when analysing $CD8^+$T lymphocytes and NK cells, clone 1.7 was the most inhibiting and clone 1.10 the most for $CD4^+$ T cells. These differences between clones in their ability to decrease lymphocyte proliferation reached statistically significant levels (post-hoc multiple comparison) in the case of NK cells for clone 3.10, *vs* clone 1.7 (p=0.0084), clone 1.22 (p=0.0112) and clone 1.10 (p=0.0279), but not significant in total and $CD3^+$ lymphocytes for clone 3.10 *vs* clones 1.22 and 1.7 (see table from Figure 8). Therefore, the individual analysis of clones allows us to distinguish three general trends with regard to their ability to inhibit lymphocyte proliferation. On one hand, clone 1.10, 1.22 and 1.7 seem to present a similar behaviour. On the other, clone 3.10 presented a significantly different behaviour from the aforementioned clones. Finally, clone 3.5 had an intermediate effect. We removed the data from PBL in order to mainly focus on the differences between clones; however the results from the ANOVA and post-hoc tests did not provide any additional information.

DISCUSSION

MSC are multipotent progenitor cells that, besides representing an attractive tool in regenerative medicine, also possess interesting immunomodulating properties, rendering them useful in surgical procedures or for the treatment of autoimmune/autoinflammatory diseases. In this respect, the aim of this approach would be to reduce the use of immunosuppressive drugs and therefore the number of side effects and even to modify the severity of the disease. Indeed, several studies have shown that MSC are capable of suppressing T-cell and NK responses and to modify B-cell and dendritic cell differentiation, maturation, and function, without being inherently immunogenic [16]**.** This latter point is extremely important, as it demonstrates that these cells may be isolated from different donors and further expanded *in vitro* so they can then be used in allogeneic recipients under cell therapy protocols. Numerous

researchers have already demonstrated the successful recruitment and multi-organ engraftment capability of infused MSC in various animal models and also in human clinical trials [30-33]. Some authors have shown that when MSC are cultured as single-cell-derived colonies, each individual colony shows variable degree of plasticity in terms of tissue differentiation. This indicates that certain colonies are only capable of differentiating towards a unique lineage while others have a higher differentiation potential [27, 34]. It has to be mentioned that the analysed clones presented certain heterogeneity in their differentiation potential towards the osteogenic and adipogenic lineages [27]. In this respect, clones 1.22 and 1.7 differentiated into both lineages, clone 3.5 displayed only differentiation to the osteogenic lineage and clones 1.10 and 3.10 were not capable to differentiate. The focus of this work was to analyse if this plasticity is also present in the MSCs' immunomodulating properties, and if so, would allow us to select the most adequate clone(s) for each specific case to treat different pathologies. To this end, we sought to analyse the possible differences that may exist between several *in vitro* expanded hASC clones obtained from two healthy donors, regarding their membrane phenotype expression, cytokine profile and their ability to inhibit lymphocyte proliferation *in vitro*.

In this context, the conflicting results that may be observed in the literature when using MSCs are possibly due to the different isolation protocols and culture conditions, resulting in a heterogeneous cell population. Furthermore, some works have shown that the source of harvested MSC plays a role in lineage commitment [35, 36]. Even though there is evidence that MSCs are capable of changing their expression pattern of surface markers depending on the culture time [16], it is important to highlight that all of our clones in this work have been cultured the same amount of time and passages. Regarding membrane phenotype expression, our clones were not significantly different, although a few differences could be detected. Briefly, clones 3.X seemed to be more homogenous than clones 1.X with respect to their

surface antigen expression. This homogeneity, apart from being an indicator of the degree of purity of the clone, could be useful for cell therapy protocols, since the expected results should be in principle more reliable than when using more heterogeneous populations. On the other hand, individual analysis of the clones also showed that there are differences in the expression of several surface antigens. Clones 1.10 and 1.22 expressed lower amounts of antigens than the other three clones, with 1.22 expressing the lowest amounts for all of the antigens. Clones 3.10 and 3.5 expressed more CD105 than the other clones and clone 1.7 expressed the highest amounts of CD73 and CD44. The balance between membrane antigens mainly involved in activating functions such as CD44 or CD90 and markers such as CD73 or CD105 which are more involved in suppressive functions, can determine, together with additional mechanisms, the final immunomodulating properties of the clones. Therefore, from our point of view, when using cell therapy protocols, apart from granting homogeneity as previously mentioned, this antigen balance should also be an additional factor to be considered before choosing a clone for these purposes.

Several studies carried out with whole MSCs demonstrate the ability of these cells to express and/or secrete a broad variety of cytokines that have been shown to be involved to some extent with their immunomodulating properties [37]. Several authors have described two morphologically distinct MSC types according to their *in vitro* cytokine release: MSC1 which mainly secretes pro-inflammatory cytokines and MSC2 which mainly secretes immunosuppressive cytokines [37]. Therefore, previous analysis of MSC1/MSC2 balance when working with heterogeneous preparations of MSCs is an indirect tool to obtain a more uniform cell culture to be used in possible cell therapies. By measuring the cytokine profiles of expanded MSC clones instead of using heterogeneous populations, we can obtain the same information about the potential immunomodulating activities of each clone and at the same time will allow us further improve the aforementioned desirable uniformity expected for cell

therapy protocols. After analysing the cytokine profile of our clones, we have also discovered some interesting differences. In fact, all of our clones were capable of spontaneously releasing high levels of IL-6 and low to moderate levels of IL-8, with 1.7 and 3.X clones producing the highest amounts of these cytokines. In general, these results were coincident with the pattern of cytokine gene expression and the corresponding DNA methylation profile. Regarding kinetics, clones 1.7, 1.22 and 3.10 seemed to present the fastest kinetics and clones 1.10 and 3.5 the slowest. Although IL-6 is a pleiotropic cytokine with both pro-inflammatory and anti-inflammatory effects, the former effect usually predominates [38, 39] and has been directly linked to the pathophysiology of diverse autoimmune diseases, in particular rheumatoid arthritis [40]. IL-8 is also involved in pro-inflammatory activities [41]. Kinetics and secretion levels after LPS stimulation from the rest of the analysed cytokines are described in detail in the Results section and slightly differ depending on the cytokine. Briefly, clones 3.X were once again the cells producing the highest amounts of the other pro-inflammatory cytokines such as IL-1β and at the same time expressed the lowest amounts of well-known anti-inflammatory Th2 cytokines such as IL-4 and IL-5. On the other hand, clones 1.10 and 1.22, despite being capable of producing very low levels of several pro-inflammatory cytokines such as TNF-α, TNF-β or INF-γ, are also the clones producing the highest amounts of IL-4 and IL-5 (low to moderate levels), being most abundant in clone 1.22. Altogether, we can conclude that in general terms clones 3.X show a more pro-inflammatory profile while clones 1.10 and 1.22 are more suppressive. Regarding the behaviour of clone 1.7, although belonging to 1.X group it seems however to be more similar to 3.X clones.

Several authors have described an ability of whole heterogeneous MSC populations to inhibit proliferation and activation of different allogeneic immune subsets without being immunogenic themselves [42]. These results offer an insight into the interactions between allogeneic MSCs and immune cells and provide mechanisms that may be involved with the *in*

vivo MSC-mediated induction of tolerance, which could be used therapeutically for reducing graft rejection or graft-versus-host-disease (GVHD) and for modulating inflammation in autoinflammatory/autoimmune diseases. Nevertheless, these studies often present contradictory results, possibly due to the heterogeneity of MSCs populations used in the different analyses [35]. In an attempt to maximally reduce this heterogeneity, we have analysed here the ability of different ASC clones to inhibit proliferation of several lymphocyte subsets. Similar to what we have described for membrane phenotype and cytokine profile, significant differences between clones 1.X and 3.X have been found. In general terms, 1.X clones are altogether more suppressive than 3.X clones for the different analysed lymphocyte subsets. However, a descriptive individual analysis shows that clone 1.22 was the most suppressive for total and $CD3^+$ lymphocytes, clone 1.7 for $CD8^+$ T lymphocytes and NK cells and clone 1.10 for $CD4^+$ T cells. Clone 3.10 was the least suppressive in all cases, followed by clone 3.5. These results are consistent with the previous data referred in this study. Indeed, clone 1.22, which was the most suppressive, expressed the lowest amounts of surface antigens, secreting low amounts of some of the most powerful pro-inflammatory cytokines and high amounts of anti-inflammatory cytokines. The opposite is true for 3.X clones, which expressed more surface antigens, secreted more pro-inflammatory cytokines and less anti-inflammatory cytokines while at the same time were the least suppressive.

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ISCIII) (www.cegen.org).

CONFLICT OF INTEREST DISCLOSURE

The authors declare that there is no conflict of interest related to the present work.

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FIGURE LEGENDS

Figure 1. hASCs single-cell derived clones present differences in their membrane phenotype Data shows the membrane phenotype from the different clones in one representative experiment of the three carried out by direct immunofluorescence and flow cytometry. Forward vs. Side scatter gate (A). Percentage of hASCs clones 1.10 (B), 1.22 (C), 1.7 (D), 3.10 (E) and 3.5 (F) expressing (grey) or not expressing (white) CD73, CD90, CD44 or CD105 surface antigens.

Figure 2. Descriptive quantitative analysis of surface antigen expression of the hASCs clones. **A)** Percentage of cells coexpressing CD90, CD73, CD44 and CD105 membrane antigens. Results are expressed as mean ± SEM (n = 3). **B)** Data shows differences in median fluorescence intensity (MFI) values of CD34, CD73, CD90 and CD105 for clones 1.10, 1.22, 1.7, 3.10 and 3.5 in one representative experiment of the three carried out by direct immunofluorescence and flow cytometry.

Figure 3. Cytokines spontaneously secreted by the different hASCs clones (A and B) or after stimulation with LPS (C). A and B) Different kinetics of released IL-6 (A) and IL-8 (B) cytokines between hASCs clones. Clones were seeded ($2x10^4$ cells per well) into flat-bottom 96-well plates and incubated for 96 h. A whole kinetics was performed for each cytokine at 2, 4, 8, 12, 24, 48, 72 and 96 h of culture. The presence of both cytokines in supernatants was analysed by Flow Cytometry through the *Flowcytomix*^{TM} *Multiplex Test* (eBioscience). **C) hASCs clones express different Th1/Th2 cytokine profiles.** Clones were seeded

($2x10^4$ cells per well) into flat-bottom 96-well plates, stimulated with LPS (0,2 μg/ml) and incubated for 96 h. Concentration of each cytokine in supernatants was measured at different times by Flow Cytometry through the *Flowcytomix™ Multiplex Test* (eBioscience).

Figure 4. Expression profile of the indicated cytokines (A) and methylation degree of the corresponding genes (B) in non-stimulated hASCs. A) Cytokine gene expression for IL-6, IL-8, IL-4, TNF-α and IL-10 was assessed by RT-PCR under conditions indicated in Materials and Methods. No RT-PCR signal was detected for IL-1β, IL-2, IL-5, IFN-γ and TNF-β (not shown). Positive control (+) corresponds to human lymphocytes. **B)** Methylation pattern for IL-6, IL-8, IL-4, TNF-α, IL-10, IL-1β, IL-2, IL-5, IFN-γ and TNF-β was quantified according to a 0 (green)-1 (red) scale. Microarray-based DNA methylation profiling was performed with Infinium HumanMethylation27 BeadChip arrays (see Materials and Methods).

Figure 5. Clustering heatmap including the methylation values for all GpC clusters of all genes related with cytokine signaling for the different clones. Microarray-based DNA methylation profiling was performed on all hASCs clones with Infinium HumanMethylation27 BeadChip arrays as indicated in Materials and Methods.

Figure 6. Expression profile of the indicated cytokines in clone 1.10 incubated in the presence of 5-aza-2'-deoxycytidine. IL-6, IL-8, IL-4, TNF-α and IL-10 gene expression was assessed by RT-PCR in clone 1.10 incubated in the absence

(-AZA) or in the presence (+AZA) of 20 μM 5-aza-2'-deoxycytidine as indicated in Materials and Methods. Positive control RT-PCR signals (+) corresponded to human lymphocytes.

Figure 7. hASCs clones obtained from different donors inhibit lymphocyte proliferation in different degree. The clones were cocultured with PBMC obtained from 7 unrelated donors in a ratio PBMC: hASCs 10:1 and stimulated with PHA (10 μg/mL) for five days. Proliferation of PBMC and corresponding subsets was significantly inhibited (***, $p<0.0001$). Comparing 1.X to 3.X clones, proliferation inhibition exerted by clones 1.X (grey bars) was significantly different from the inhibition exerted by clones 3.X (white bars) for total lymphocytes and $CD3^+$T lymphocytes (**, $p< 0.005$), and for $CD4^+$T lymphocytes and NK cells (*, $p< 0.05$). No significant effect was observed for CD8+ T lymphocytes ($p=0.349$). Results are express as mean ± SEM for at least n=14.

Figure 8. Descriptive analysis of the effect of hASCs clones on lymphocyte proliferation. Clones 1.X (grey bars) and 3.X (white bars) exerted a significant inhibition of PBMC proliferation in co-culture experiments compared to PBMC cultured alone (black bar) (***, $p < 0.000$). The inserted table indicates the statistical analysis. Results are express as mean ± SEM for n=7.

Figure 1

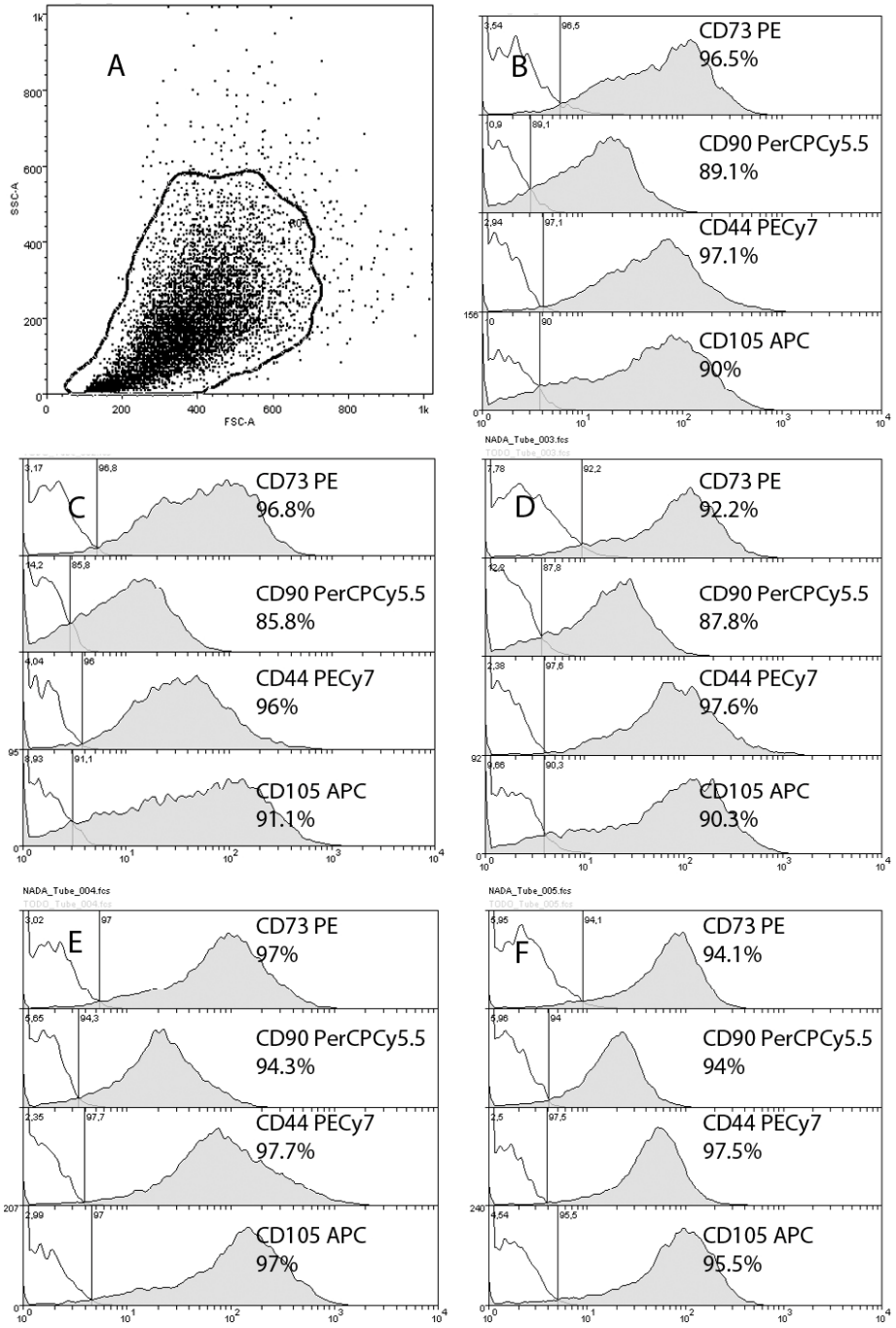


Figure 2

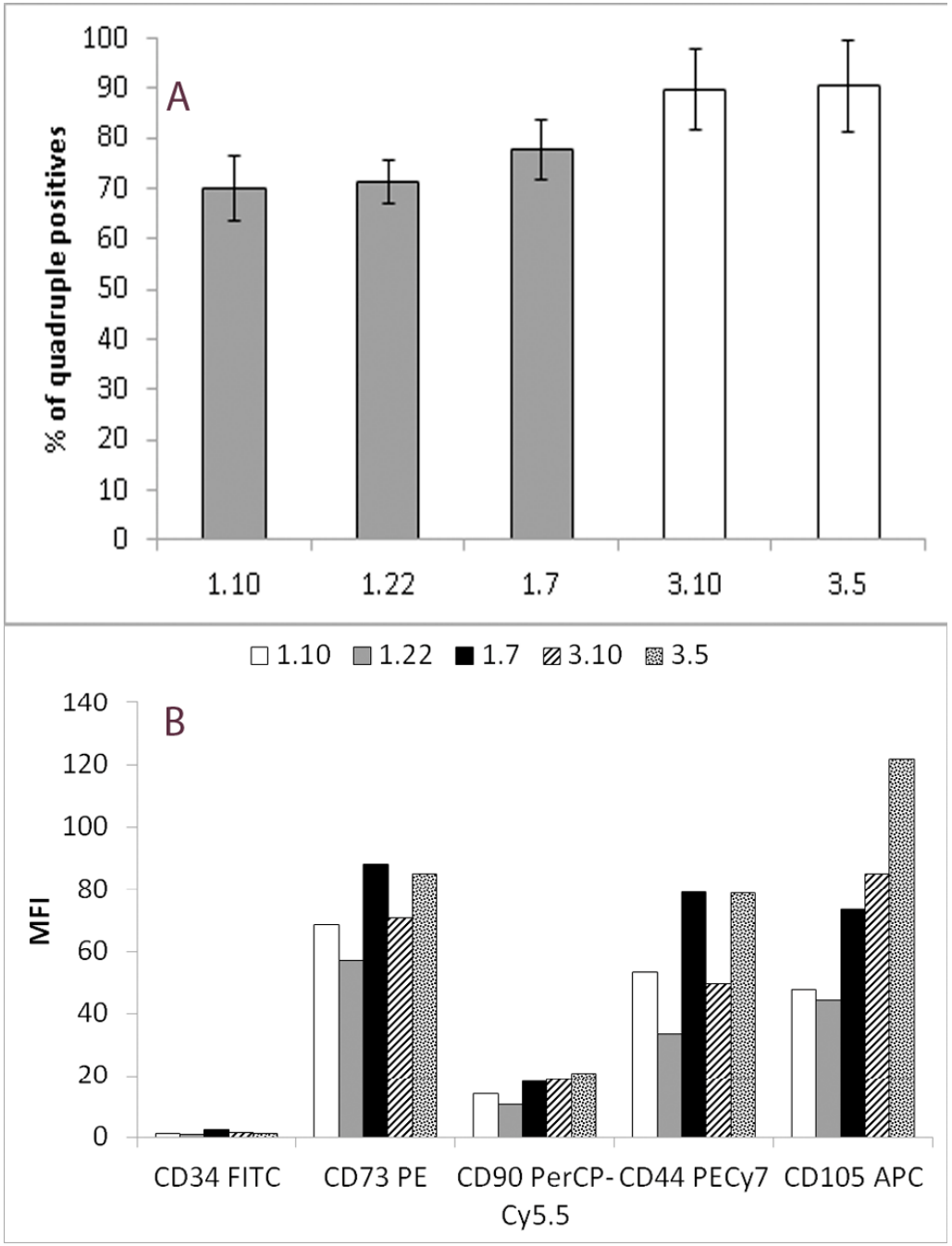


Figure 3

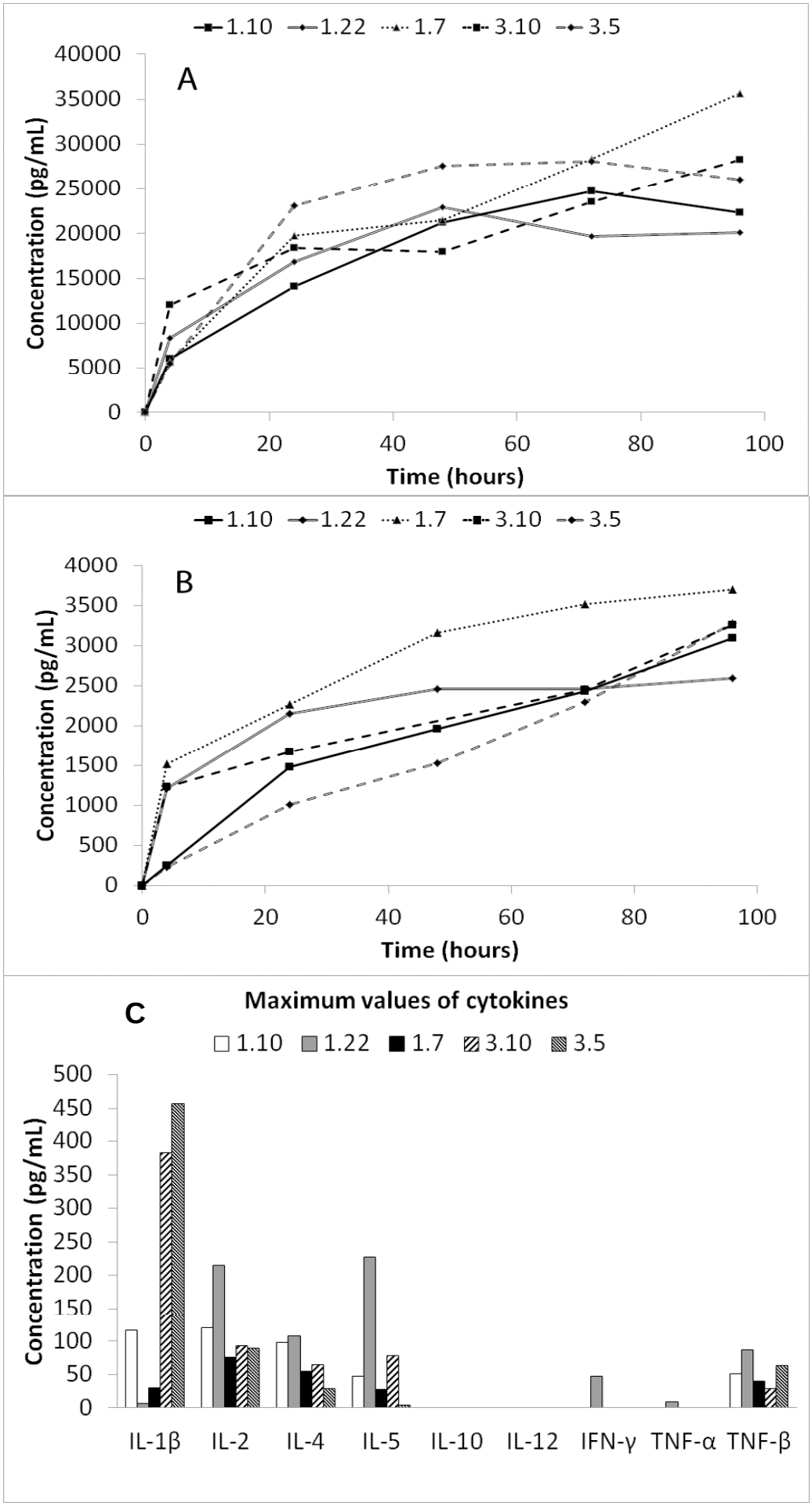


Figure 4

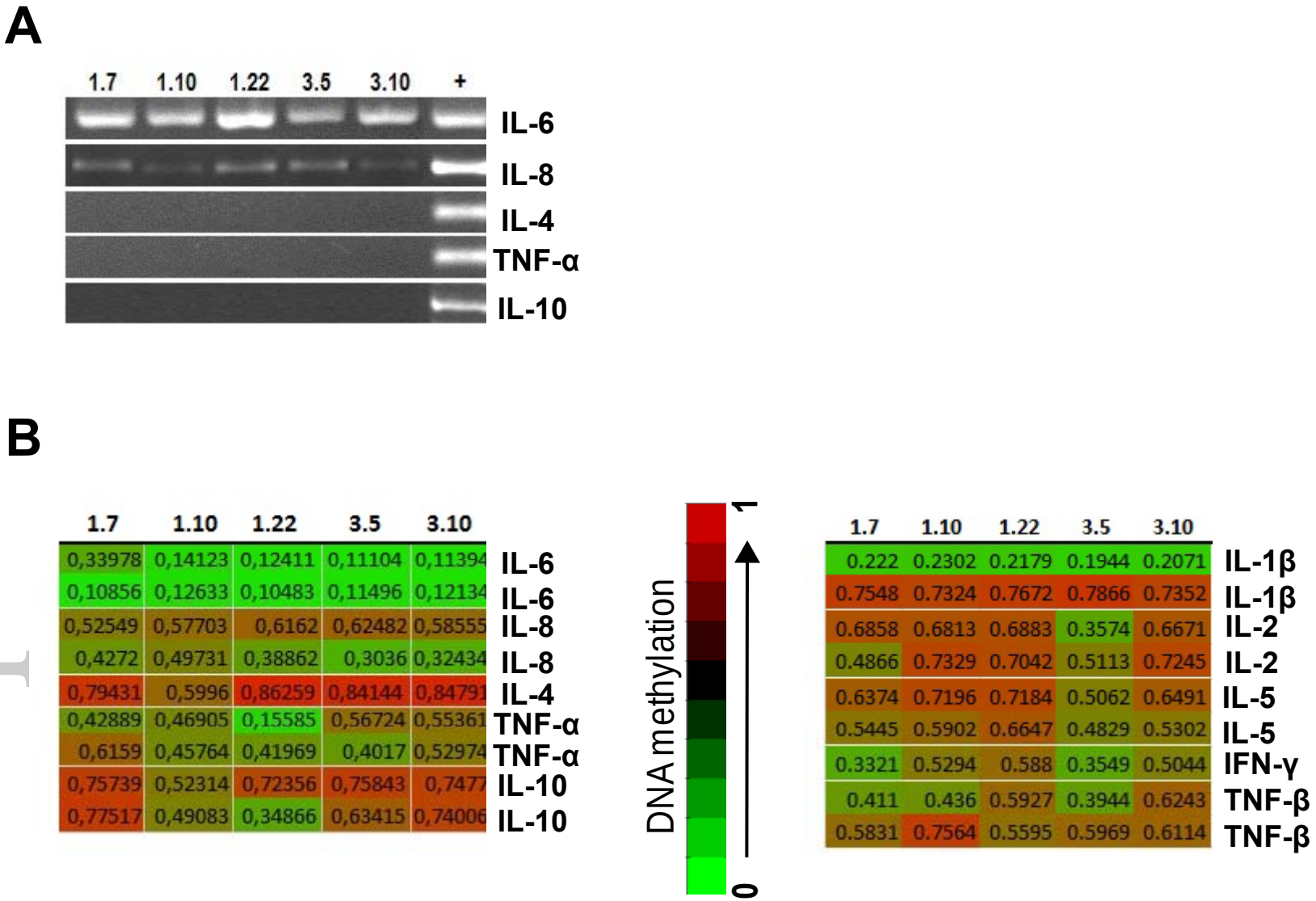


Figure 5

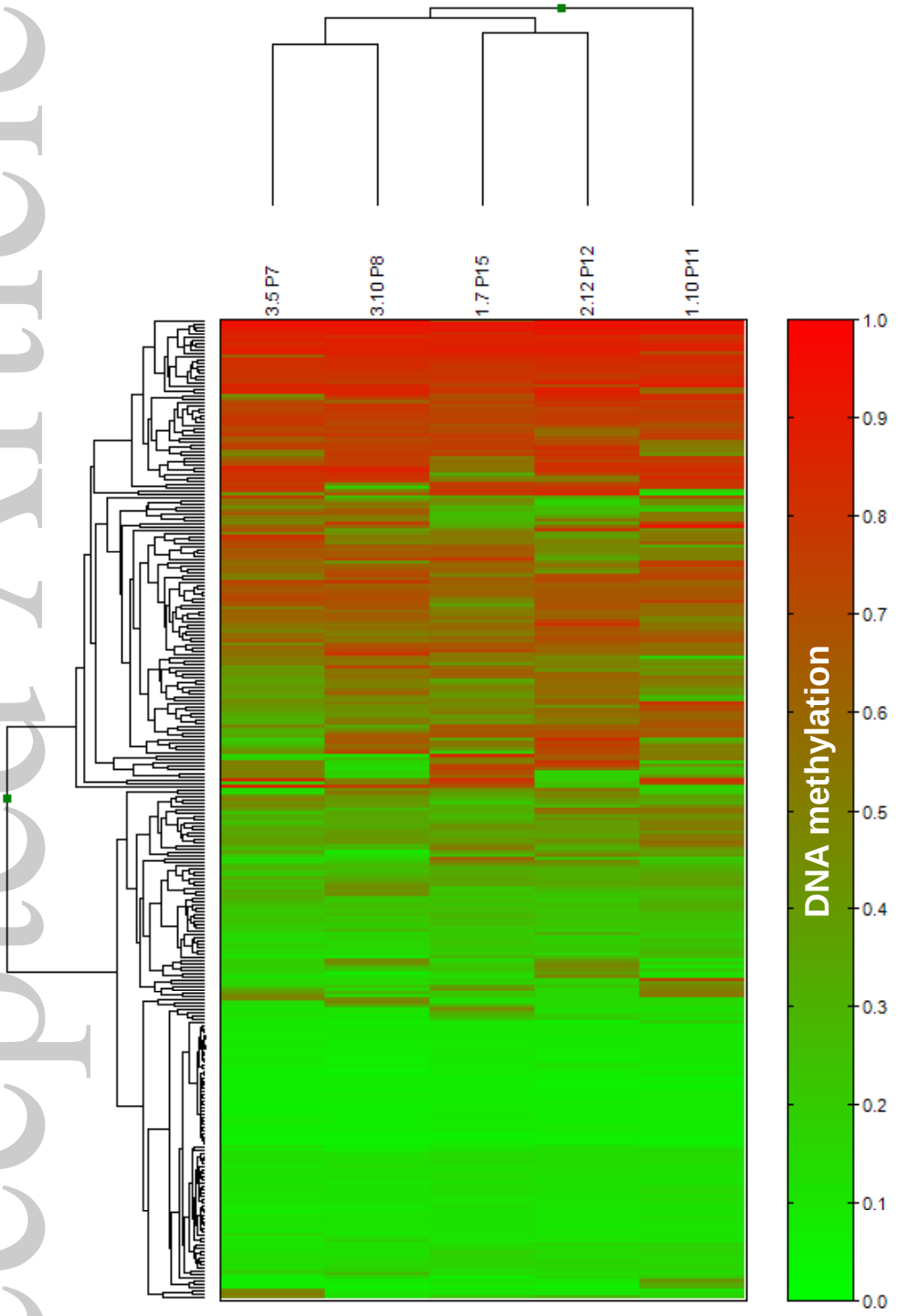


Figure 6

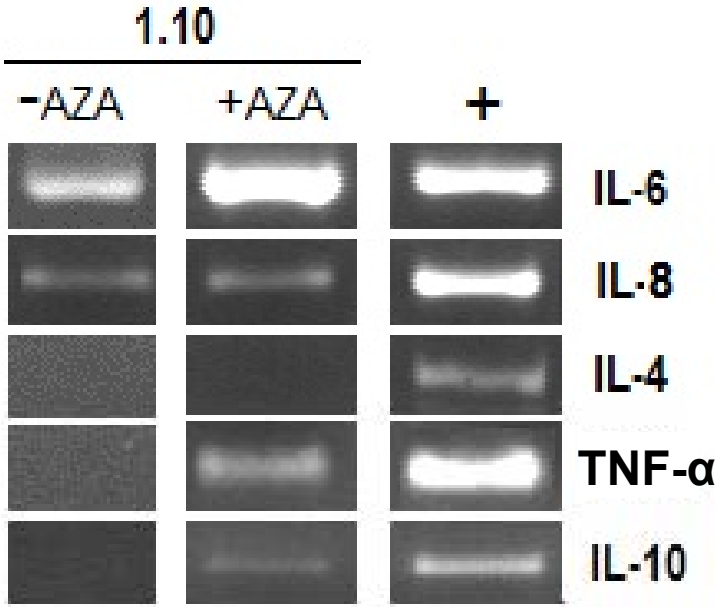


Figure 7

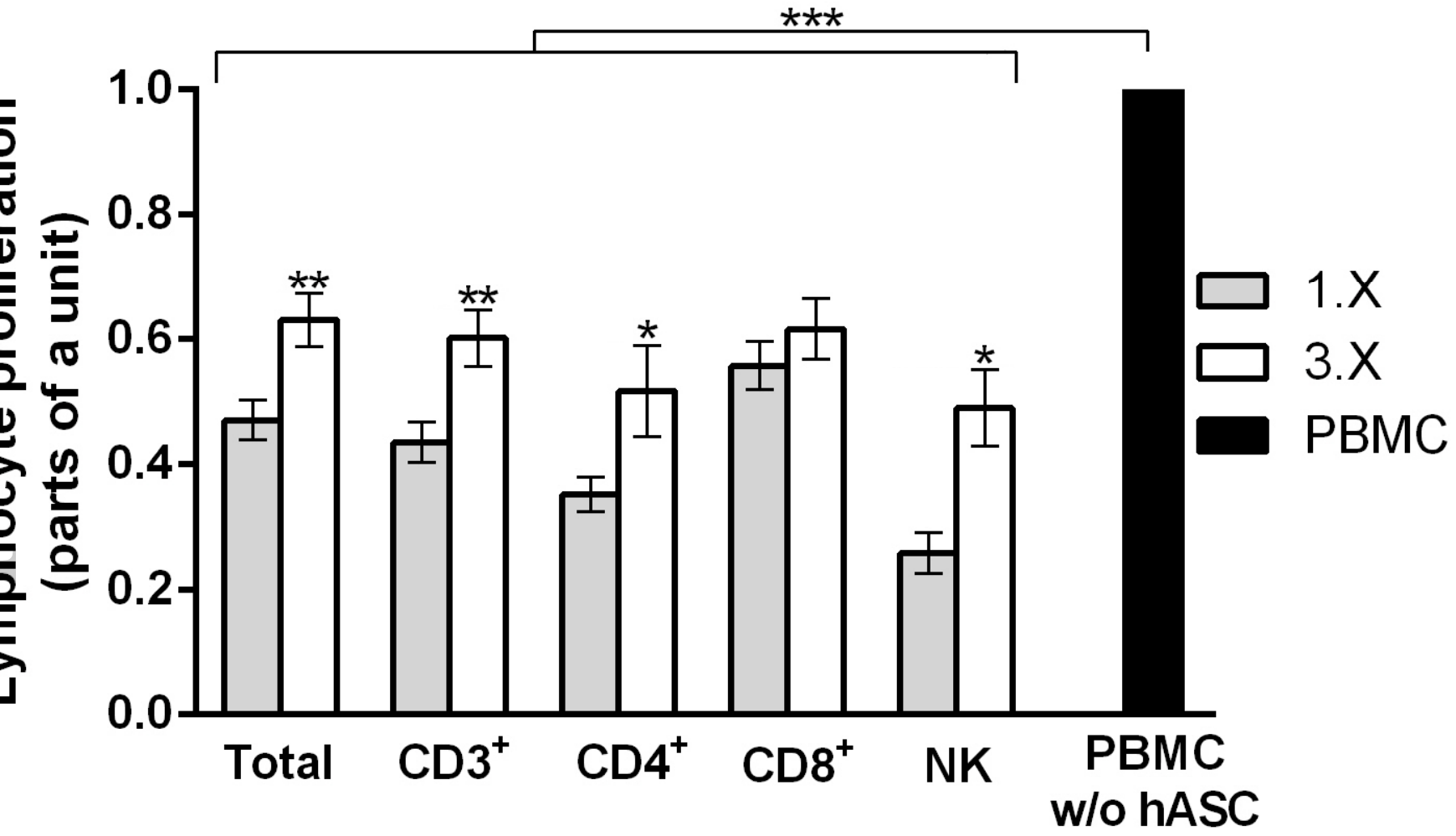
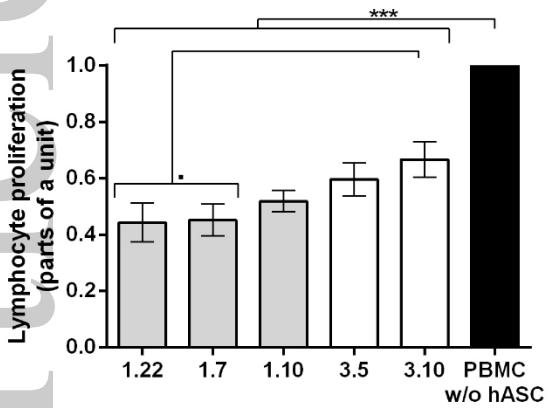
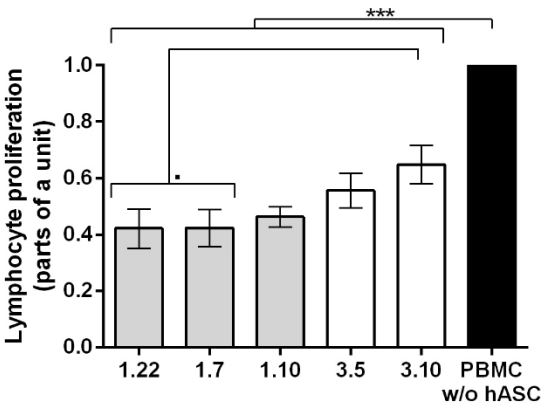


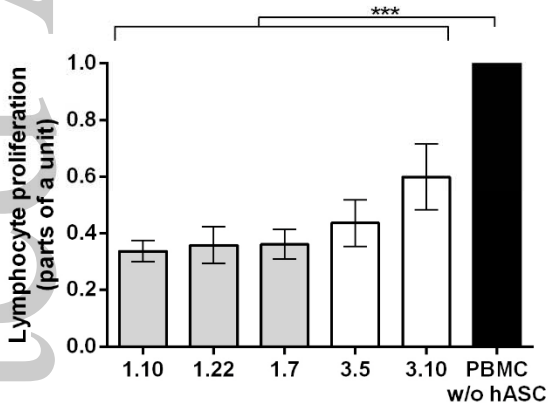
Figure 8



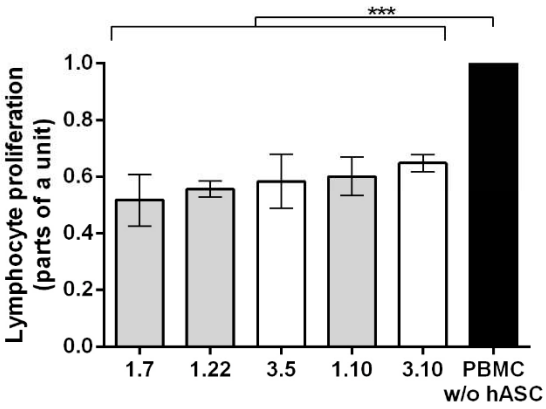
Total Lymphocytes



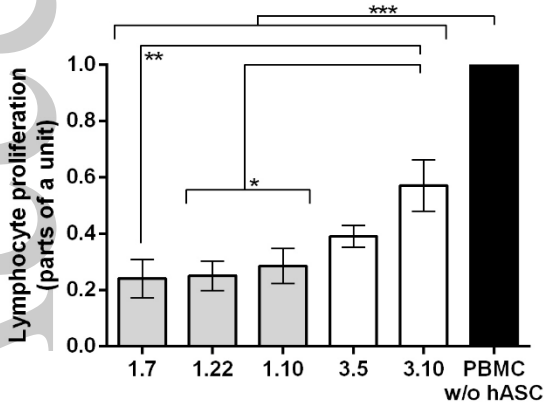
CD3 Lymphocytes



CD4 Lymphocytes



CD8 Lymphocytes



NK Lymphocytes

Marker		*Differences*	*Sig*
TOTAL	ANOVA	$1.5 \cdot 10^{-7}$	***
Post-Hoc:			
All clones vs PBMC	0.000		***
1.22 vs 3.10		0.0565	.
1.7 vs 3.10		0.0728	.
CD3	ANOVA	$1.43 \cdot 10^{-7}$	
Post-Hoc:			
All clones vs PBMC	0.000		***
1.22 vs 3.10		0.0761	.
1.7 vs 3.10		0.0806	.
CD4	ANOVA	$1.32 \cdot 10^{-5}$	***
Post-Hoc:			
All clones vs PBMC	0.000		***
CD8	ANOVA	$8.06 \cdot 10^{-5}$	***
Post-Hoc:			
All clones vs PBMC	0.000		***
NK	ANOVA	$2.07 \cdot 10^{-8}$	***
Post-Hoc:			
All clones vs PBMC	0.000		***
1.10 vs 3.10		0.0279	*
1.22 vs 3.10		0.0112	*
1.7 vs 3.10		0.00084	**