

Paracrine activity of adipose derived stem cells on limbal epithelial stem cells

Bartosz Sikora (✉ bsikora@sum.edu.pl)

Medical University of Silesia in Katowice

Aleksandra Skubis-Sikora

Medical University of Silesia in Katowice

Agnieszka Prusek

Medical University of Silesia in Katowice

Joanna Gola

Medical University of Silesia in Katowice

Research Article

Keywords: cornea regeneration, wound healing, inflammation, mesenchymal stem cells, interleukins

Posted Date: June 15th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-551083/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Limbal stem cells deficiency (LSCD) is an eye disease caused by the loss of stem cells in the corneal limbus as a succession of an injury due physical, biological, or chemical agents. Current therapies of LSCD are focused on the transplantation of donor corneas or tissue equivalents produced from autologous limbal stem cells. Every year there are waiting millions of people for the cornea transplantation all over the world and the list of waiting patients is growing due to the relatively low number of cornea donors. On the other hand, the transplantation of tissue or cells into the recipient's body is associated with the higher risk of possible side effects. The possibility of the application of an indirect treatment using the properties of the paracrine activity of stem cells, would be beneficial for the patients with transplant failures. This study was to evaluate the paracrine effect of mesenchymal stem cells derived from adipose tissue (ADSC) on the viability of limbal epithelial stem cells (LESC). The paracrine effect was assessed by treating LESC with conditioned medium collected from ADSC culture. Cell viability, cytotoxicity, apoptosis and proliferation were evaluated using *in vitro* assays in standard conditions and induced inflammation. After the exposure to the examined conditions, the expression of genes related to pro- and anti-inflammatory factors was evaluated and compared to the secretion of selected cytokines by ELISA test. Moreover, the changes in LESC phenotype were assessed using of phenotype microarrays. Our findings suggest that paracrine activity of ADSC on LESC promotes its proliferation and mitigates the adverse impact of induced inflammation.

Introduction

Cornea is front, transparent part of eye responsible for transmission and refraction of light. It possesses high self-regenerative ability due to the presence of limbal epithelial stem cells (LESC) what makes the cornea easy to be transplanted. LESCs are localized in corneal limbus which is a thin, transparent cell zone in the junction between cornea, sclera and conjunctiva [1]. Limbal Stem Cell Deficiency (LSCD) may occur due to chemical or thermal burns and physical destruction. LSCD is also a congenital disease like aniridia-associated keratopathy (AAK), corneal leukoma or Fuchs' dystrophy [2–4].

Cornea is one of the most frequently transplanted organs. Unfortunately, despite the possibility of transplantation, insufficient number of donors in comparison to number of recipients is still a huge problem of transplant medicine. Statistical report of Eye Bank Association of America (EBAA) showed that 85441 corneal transplantations were done in year 2018 [5]. Reported cases of corneal transplantations in 2019 reached 85601. In 2020 due the worldwide pandemic of COVID-19 reported procedures dramatically decreased to the number of 66278 surgeries. The pandemic has influenced on performing many surgical procedures and the implemented sanitary restrictions in most countries all over the world had slower healthcare system what appeared in lower number of all surgical procedures. Instead of the successfully ended cornea transplantation reported by EBAA, every year there is a huge number of waiting patients for the corneal transplantation all over the world. In 2016 it was reported about 12.7 million of waiting patients [6].

Treatment of the corneal disorders may embrace partial or whole cornea transplantation. Unfortunately, the number of patients waiting for the graft indicates that there are not enough cornea donors, thus newer and newer medicinal products for corneal reconstruction are being introduced. When other treatment fails, standard surgical procedure of damaged cornea replacement with a donor tissue is being displaced by artificial cornea transplantation [7–10]. As well the use of xenogeneic corneas is being considered in the absence of cornea donors [11–13]. However, each medicinal product of such matter is produced with active stem cells usually harvested from small biopsy taken from the healthy limbal region of patients' eye. Such a biopsy could potentially broaden stem cells deficiency and cause extensive damage to the corneal epithelium. On the other hand, not all the corneal injuries require immediate replacement. New strategies may focus on improving residual limbal stem cells viability and supporting them with factors or drugs aimed to activate their proliferation and thus tissue reconstruction. Therefore, new strategies on corneal treatment, based on supporting the residual stem cells should be developed. Regarding these concerns we assume that new strategy of corneal treatment which will be based on supporting residual healthy stem cells niche in patients' eye will be the future therapy of limbal stem cells deficiency.

Mesenchymal stem cells (MSCs) are somatic cells with wide regenerative properties. Research focus on using them e.g. in bone and cartilage failures regeneration or cardiovascular disease therapies. One of the common sources of MSCs is adipose tissue. Adipose tissue is a rich source of mesenchymal stem cells. Its collection is an easy procedure and the probable amount of stem cells which can be gathered during the isolation procedure is high. Considering other sources of mesenchymal stem cells, liposuction is burdened with low risk of complications in opposite to the bone marrow biopsy. Mesenchymal stem cells, besides its plasticity, exhibit immunomodulatory abilities by secreting numerous cytokines [14, 15]. ADSC affects neighboring cells also via secretion of growth factors [16, 17] and extracellular vesicles [18–20]. Secretome of ADSC promotes proliferation and reduces inflammation [21, 22]. MSCs secrete anti-scarring factors as KGF, SDF-1, MIP-1a, MIP-1b, anti-apoptotic agents as STC-1, SFRP2, TGF- β 1, VEGF and HGF, molecules associated to angiogenesis like VEGF, TGF- β 1 and mitogenic factors as TGF- α , TGF- β , HGF, IGF-1, FGF-2 or EGF [15, 23]. Research indicated that MSCs secretome act positively on corneal epithelium [24]. It suggests that ADSC can be used clinically in the treatment of LSCD.

Our experiment evaluates the paracrine activity of ADSC on LESC'S dysfunction model *in vitro*. The effect of ADSC'S secreted factors on the proliferation, metabolic activity, and transcriptome of limbal stem cells was analyzed under conditions of induced inflammation using bacterial lipopolysaccharide (LPS).

Material And Methods

Experiment

Paracrine activity of ADSC was assessed in an *in vitro* model of LESC dysfunction. Experiment was conducted in two aspects. First, the direct influence of conditioned medium (CM) from ADSC on LESC activity was assessed. The examined cells were compared to the control cells cultured in standard medium (SM). Then, LESC were pretreated with LPS to induce inflammation and again cultured in CM to

assess its influence under the condition of induced inflammation (CM_LPS group). The control cells were cultured in SM after analogous pretreatment with LPS (SM_LPS group).

Cell culture conditions

ADSC (PT-5006) were obtained from Lonza company (Switzerland) and LESC were provided by courtesy of Department of Microbial Biotechnology and Cell Biology, Faculty of Science, University of Debrecen (Hungary) where they were isolated and identified [25]. ADSC and LESC were cultured in a Dulbecco's Modified Eagle Medium (DMEM, Lonza, Switzerland) with 10% of fetal bovine serum (FBS, EuroClone, Italy) and 1% of antibiotics: amphotericin B with penicillin-streptomycin (Lonza, Switzerland) at 37°C in a 5% CO₂ incubator (Direct Heat CO₂; Thermo Fisher Scientific, USA). The medium was changed every 48 h. Identification of ADSC was made with real time RTqPCR analysis of genes: *CD73*, *CD90* and *CD105* and flow cytometry with Human Mesenchymal Stem Cell Marker Verification Multi-Color Flow Cytometry Kit for the analysis of surface proteins: CD73, CD90 and CD105 (R&D Systems, USA). Cell fluorescence was measured with FACS Aria 2 (Becton Dickinson, USA) and the data was analyzed using software (Becton Dickinson, USA) (not shown).

Conditioned medium preparation

CM was collected from 24 h culture of ADSC at 60–70% confluency. It was centrifuged at 3500 rpm for 5 min to pellet down dead cells and cell debris. Then supernatants were filtered through 0.22 µm filters. The filtered supernatants were diluted with a SM (DMEM, 10% FBS, 1% antibiotics) in a ratio 1:1. [26]. The SM for the experiments was prepared simultaneously in analogous manner. First portion was incubated for 24 h in clean culture dishes and then it was centrifuged, filtered, and diluted in a 1:1 ratio with fresh portion of medium.

MTT assay

3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich, USA) was used to evaluate the viability of the cells after exposure to LPS to determine the optimal concentration for cells stimulation [27]. Absorbance referring to the concentration of formazan was measured using Wallac 1420 VICTOR plate reader (Perkin Elmer, USA) after 24 h of exposure to 0,1 µg/ml; 0,2 µg/ml; 0,5 µg/ml; 0,7 µg/ml; 1 µg/ml and 2 µg/ml of LPS and compared to control. All groups were analyzed in six replicates.

Inflammation inducement

To mimic the inflammation, LESC were pretreated with LPS (lipopolysaccharide from *E. coli*, Sigma-Aldrich, USA) at concentration of 2 µg/ml for 24 hours.

ApoTox-Glo Triplex Assay

To measure cell viability, cytotoxicity and apoptosis in the samples, the ApoTox-Glo Triplex Assay (Promega, USA) was used according to the manufacturer's instructions. Viability, cytotoxicity, and apoptosis were evaluated after 24 h of cells exposure to examined conditions. LESC were cultured in SM,

CM after pretreatment with LPS (groups: SM_LPS, CM_LPS) and compared to standard conditions (groups: SM, CM). Viability and cytotoxicity were measured by the fluorescent signal, which was emitted due to the cleavage of the added substrates glycyl-phenylalanyl-aminofluorocoumarin (GF-AFC) for viability and bis-alanyl-alanyl-phenylalanyl-rhodamine 110 (bis-AAF-R110) for cytotoxicity by specific proteases. GF-AFC enters the cells where it is cleaved via the live-cell protease activity to generate a fluorescent signal that is proportional to the number of living cells. AAF-R110 was used to measure the dead-cell protease activity, which was released from the cells that have lost membrane integrity. Both substrates have different excitation (400 nm, 485 nm) and emission (505 nm, 520 nm) spectra. Apoptosis was measured by the addition of a Caspase-Glo 3/7 Reagent. It is a luminogenic substrate that contains the tetrapeptide sequence DEVD in the reagent to assess caspase activity, luciferase activity and cell lysis. Fluorescence and luminescence were measured using a plate reader Triad LTMultimode Detector (Dynex Technologies, USA) [28]. All groups were analyzed in six replicates.

Scratch assay

LESC'S proliferation rate was analyzed in all study groups (SM, SM_LPS, CM, CM_LPS). For this purpose, a scratch assay (wound healing test) was done. The LESCs were grown in standard condition to 80% confluence and then cells were exposed to LPS for 24 hours to induce the inflammation. Control cells were cultured without LPS. After reaching the full confluence, the scratch was made using 200 μ l pipette tip and cells were washed with PBS to remove non-adherent cells. After that medium was replaced for SM or CM [29]. Then the scratch area was photographed with an Olympus IX81 microscope (Japan) with a DP70 Olympus camera (Japan) and the wound area was measured using Image J software in time points: 0 h, 6 h, 12 h, 24 h, 36 h. All groups were analyzed in six replicates.

Phenotype microarrays (PMM)

The phenotype of LESCs was assessed under the influence of SM, CM and SM with the addition of LPS (SM + LPS) with phenotype microarrays (PMM, Biolog, USA). Phenotype microarrays are 96-well microplates coated with different substrates in appropriate solvents e.g. ions, hormones and metabolic effectors. PMM show how cells metabolism, growth and productivity is affected by these agents. In order to the composition of substrates located on microarray, the PMM7 and PMM8 plates were used. These arrays are microplates coated with growth factors (e.g. IGF, FGF, PDGF), hormones (e.g. leptin, somatotropin, calcitonin, TSH) and cytokines (e.g. IL-1 β , IL-6, IL-8, TNF α , IFN γ). This method enables the demonstration of cell preferences in the uptake of substrate added to the well of microarray. It also gives an information about possible cytotoxic effect of single compound. For this method cells previously were cultured in examined media for 24 h. Then, cells were collected by trypsinization and seeded on microarrays at the density of 10000 cells per well. For the each medium (SM, CM, SM + LPS) one microarray of each type was used. LESCs were cultured on microarrays for 24 h and after this period a dedicated tetrazolium salt (provided by the producer) was added to the wells for 3 h of further incubation. Added salt was reduced to soluble formazan what appeared in color-change reaction. The measured absorbance value corresponded to cell viability. The absorbance was measured with a Wallac 1420 VICTOR plate reader (Perkin Elmer, USA) at 590 nm [30].

Quantitative real-time polymerase chain reaction assay (real time RTqPCR)

Total RNA was extracted from the cells using a TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. The nucleic acid concentration was determined using a MaestroNano Spectrophotometer (MaestroGen, Taiwan). The expression of mRNAs related to inflammation (*IL6*, *IL-10*, *IL-2*, *IL-1 α* , *IL-1 β* , *IFN γ*) was detected using the real time RT-qPCR technique with SYBR Green chemistry (SensiFAST™ SYBR® No-ROX Kit, Biorad, USA) and an Opticon™ DNA Engine Continuous Fluorescence detector (MJ Research Inc., USA) as was previously described [27]. For each biological replicate three technical replicates were performed.

Enzyme-linked immunosorbent assay (ELISA)

The concentration of interleukins (IL-2, IL-4, IL-10, IL-6, IL-1 α) secreted by LESC to culture medium after exposure to the examined conditions, was analyzed with the use of immunoenzymatic tests (R&D Systems Quantikine ELISA Kits, USA) according to manufacturer protocol, as was described previously [31]. Optical density (OD) was read at 450 nm using Wallac 1420 VICTOR2™ (PerkinElmer Inc., USA). For each biological replicate three technical replicates were performed.

Statistical analysis

Statistical analysis was performed using Statistica 13.0 software (StatSoft, USA). A one-way ANOVA test with Tukey's post hoc test and two-way ANOVA test or T-test were applied to evaluate any significant differences in the examined groups for normally distributed data. For non-normally distributed data Kruskal-Wallis test was applied. The level of significance was set at $p < 0.05$ for all statistical tests.

Results

Inflammation inducement

Viability was evaluated after 24 h of LPS treatment at following concentrations: 0.1 $\mu\text{g/ml}$; 0.2 $\mu\text{g/ml}$; 0.5 $\mu\text{g/ml}$; 0.7 $\mu\text{g/ml}$; 1 $\mu\text{g/ml}$ and 2 $\mu\text{g/ml}$ in comparison to control (0 $\mu\text{g/ml}$ of LPS). Control cells were taken as 100% (Fig. 1.). We have not observed statistically significant difference of mitochondrial activity in examined cells. However, meaningfully decreased viability was observed in 0.2 $\mu\text{g/ml}$ ($p = 0.0337$) and 2 $\mu\text{g/ml}$ ($p = 0.0009$) groups compared to cells treated by 0.5 $\mu\text{g/ml}$ of LPS. In addition, viability of 2 $\mu\text{g/ml}$ group was also lower in comparison to 0.7 $\mu\text{g/ml}$ group ($p = 0.0351$). Based on the most meaningful biological effect, which was observed, the LPS concentration of 2 $\mu\text{g/ml}$ was selected as optimal and nontoxic for the inflammation inducement in further analyses.

Effect of ADSC secretome on viability, cytotoxicity, and apoptosis of LESC

Cells treated with CM showed a significant increase in viability ($p = 0.023$) and decrease in cytotoxicity ($p = 0.033$) comparing to the control. We have not observed significant changes in apoptosis of cells cultured in SM and CM. We did not find statistically significant differences in viability, cytotoxicity and apoptosis of cells pretreated with LPS (Fig. 2).

Impact of ADSC secretome on proliferation and migration of LESC

Scratch assay was made to assess an influence of ADSC secretome on LESC which were pretreated with LPS for 24 hours. The results of microscopic observations are presented in the Fig. 3A. Wound areas were calculated as the fold change of measured surface area (total number of pixels, $N = 6$) in examined groups (CM, SM, CM_LPS, SM_LPS) after 6 h, 12 h, 24 h and 36 h compared to the initial time point (0 h). Significant differences were observed between the examined groups. The two-way analysis of variance (ANOVA) ($p < 0.05$) showed that cells treated with CM had higher proliferation rate compared to the cells grown in SM, both in standard conditions and in induced inflammation. It was noticed that cells treated with LPS showed lower proliferation rate than non-stimulated cells, however the change was not statistically significant compared to standard conditions (Fig. 3B).

Impact of ADSC secretome on phenotype of LESC

Obtained results are presented in heatmaps (Fig. 4). The pattern of heatmap for each group significantly differs what shows that the preferences of cells has changed due to the exposure of examined media. A detailed analysis showed a diverse reaction to the presence of hormones and metabolic activators (growth factors and cytokines) present on microarrays, such as: insulin, resistin, glucagon, ghrelin, leptin, gastrin, exendin-3, hGH, IGF-I, FGF-1, PDGF-AB, IL-1 β , IL-2, IL-6, IL-8, PTH, calcitonin, LH, HCG, TSH, IFN- γ , adenosine, vasopressin, prolactin, calcitriol, LH-RH, ACTH, TRH, TNF- α , and Gly-His-Lys tripeptide. LESC showed decreased activity in the presence of insulin, IL-8, and prolactin in all examined groups. Cells cultured in CM showed less activity in response to IL-6 and the Gly-His-Lys tripeptide. Whereas cells after the culture in SM showed decreased activity in response to IL-2. Finally, cells cultured in SM + LPS showed less activity in the presence of glucagon. The above results varied in lower concentrations. A detailed description of our findings is included in discussion.

Impact of ADSC secretome on the transcriptome of LESC

The expression of pro-inflammatory cytokines *IL-1 α* ($p = 0,0075$) and *IL-1 β* ($p = 0,0098$) was significantly lower in cells cultured in CM_LPS in comparison to CM. The *IL-6* mRNA level was lower in CM_LPS cells ($p = 0,0177$) versus SM. Level of *IFN γ* mRNA in SM_LPS was higher ($p = 0,0374$) than in SM group. *IL-2* and *IL-10* mRNA did not differ in analyzed groups (Fig. 5).

Impact of ADSC secretome on inflammatory cytokines secretion by LESC

The secretion of cytokines (IL-2, IL-10, IL-6, IL-1 α , IFN γ and IL-4) was quantified by ELISA. Only the presence of IL-6 and IL-1 α was detectable, the secretion of IL2, IL10, IL4 and IFN γ has not been noticed. (Fig. 6). The concentration of IL-6 was significantly lower in cells cultured in CM both in standard conditions ($p = 0,0002$) and in induced inflammation (CM_LPS) ($p = 0,0002$) compared to SM and SM_LPS groups. The concentration of IL-1 α was significantly lower both in CM ($p = 0,0006$) and CM_LPS ($p = 0,0066$) groups compared to SM_LPS. We also noticed lower level of IL-1 α in SM ($p = 0,0002$) group compared to SM_LPS. There were no differences between CM, CM_LPS and SM groups.

Discussion

The first stage of experiment included the choice of lipopolysaccharide concentration for the inducement of inflammation. The effect of a series of concentrations of LPS on LESC was assessed, however MTT assay showed no statistically significant differences between the examined groups compared to the control. Similarly Kukolj et al. [32] observed that LPS did not affect proliferation and viability of periodontal ligament stem cells and it did not change their immunophenotype and cell cycle. Kukolj et al. showed that LPS acts on differentiation potential of cells and it inhibits osteogenesis and promotes chondrogenesis and adipogenesis [32]. Cell viability may change under the influence of LPS depending on the time of incubation and its concentration [33]. Many studies suggest the application of different concentrations from very low like 0,1 $\mu\text{g/ml}$ [34]; through 0,5 $\mu\text{g/ml}$ [35], 1 $\mu\text{g/ml}$ [33] to 10 $\mu\text{g/ml}$ [33, 34] or even 24 $\mu\text{g/ml}$ [36]. Moreover, the suggested incubation time which is necessary for the induction of inflammation is diverse and takes e.g. from 2 h to 24 h [33]. Some studies indicate that a proper time to induce the inflammation in mesenchymal stem cells is 24 h with 1 $\mu\text{g/ml}$ of LPS concentration [37]. Other suggest increasing the concentration of LPS to 2 $\mu\text{g/ml}$, but shortening the incubation time to 6 h in morphologically similar cells like normal human skin fibroblasts (NHDF) [38]. In contrast, LPS at concentration of 0,1 $\mu\text{g/ml}$ was used for the induction of inflammation in macrophages for 24 h [39]. Therefore, based on numerous literature data and our findings, the concentration selected for this experiment was 2 $\mu\text{g/ml}$ of LPS.

Next, the analysis with ApoTox-Glo Triplex Assay showed that LESC viability increased after the treatment of conditioned medium from ADSC. The test showed also that the CM was not toxic to cells and as well there were no differences in cells apoptosis between groups. These results suggest that factors secreted by ADSC improve LESC'S viability. In the condition of induced inflammation, no statistically significant differences in cell viability, cytotoxicity and apoptosis were noticed under the influence of CM. These findings suggest that LPS inhibits the positive effect of ADSC'S secretome on limbal stem cells. Probably different signaling pathways were activated in the presence of LPS in the cells, not necessarily associated with the promotion of cell viability. It indicates that the addition of LPS does not significantly reduce cell activity. Probably a longer cell culture in the above-mentioned conditions could result in similar findings as in the case of cells cultured in standard conditions. However, these results show that the presence of LPS changes the activity of cellular proteases and caspase 3/7 in LECs. Similar findings were reported by Chen and his team who investigated the effect of conditioned medium from mesenchymal stem cells derived from Wharton jelly on epithelial cells. It was shown that conditioned medium increased cells proliferation and migration by activation of stress response kinase JNK (c-Jun N-

terminal Kinase) and isoform of p38 protein [40]. Farahmand et al. also confirmed these results by observing higher expression of proliferation-related genes after culture in conditioned medium [41]. Some data indicated using a conditioned medium in the therapy of e.g. arthritis. In this case, a reduction of damages in cartilage tissue was shown and an inhibition of the immune response in cells has been noted [42]. Li et al. investigated using conditioned medium as a promoter of wound healing in diabetic patients in whom this process is usually disturbed. Research showed that the use of LPS reduces the rate of migration and proliferation in keratinocytes which grown in the presence of high glucose concentration, as the disease model for analyzing the effect of type 2 diabetes on cells. It was proven that the use of CM eliminates the negative effect of LPS and also negative effect of high glucose levels [43]. Also, the high potential of using CM in the treatment of bronchopulmonary dysplasia in oxygen-induced alveolar damage model showed that MSCs support tissue with antioxidant substances which therefore can lead to new therapy [44].

Scratch wound healing assay was performed to assess the rate of cell proliferation. LESC cultured in CM compared to SM, both under standard conditions and induced inflammation, showed better proliferation rate. It was noticed that cells stimulated with LPS and cultured in CM showed lower proliferative potential compared to unstimulated cells. These results indicate inhibition of cells proliferation by LPS, which is consistent with previous findings and the literature data [43]. Zhang et al. in *in vivo* studies showed that injection of ADSC promoted wound healing in rabbits [45]. Zeppieri et al. presented that mesenchymal stem cells promote corneal wound healing in rats [46]. Similarly, Galindo et al. demonstrated that ADSC have a therapeutic effect on LSCD in rabbits [47].

Phenotype microarrays showed that LESC in all study groups (SM, CM, SM + LPS) had various activity. PMM7 and PMM8 arrays were coated with six replicates of the same compound but in variable concentration. Producer provides an information that these concentrations are increasing from left to right, but does not give the information about the number and the unit, so we do not really know how much of each compound is in the well. We have numbered these concentrations from 1 to 6, where 1 is the lowest concentration and 6 is the highest. Due to a lot of data on cells activity, the results are presented on heat maps what ensures clear readability. All readings are presented as the fold change compared to control which was taken as 100%. We noticed that the activity of LPS significantly impact on LESC viability. This group of cells became more sensitive for bigger concentrations of each compound what appeared in the highest spread of fold change values. Interestingly, we noticed that LESC cultured in SM with the addition of LPS were more viable in presence of IL-6, while LESC cultured in SM and CM showed neutral or toxic effect of this cytokine. The lowest viability in presence of IL-6 was noticed in CM. We assume that while ADSC secrete IL-6, the final concentration of this cytokine in one well could be high enough to act negatively on LESC [48–50]. Surprisingly, the highest toxic effect was observed at the concentration “4”. LESC showed decreased activity in response to IL-8 in all examined groups. The toxic effect was more readable in higher concentrations and the highest values were noticed in CM group, but there were no differences between SM. Similarly, IL-2 in higher concentrations showed a toxic effect on LESC viability in all groups, but in opposite, lower concentrations of IL-2 induced LESC activity what was mostly notable in CM group. We observed that INF γ exerted a neutral response of LESC. In SM and CM

groups we noted slightly increasing viability in direct proportion to the INF γ concentration. LESC cultured in SM + LPS showed decreased viability in the presence of INF γ .

Phenotype microarrays brought plenty of information which could help composing supplementation of culture media to improve cells viability. These factors could be potentially cross-linked in biopolymer scaffolds adapted for carrying ADSC to strengthen the response to secreted factors. However, further studies on the influence of presented factors are needed for better understanding of variable effect on LESC activity.

The last stage included the assessment of mRNA expression and cytokine secretion of pro and anti-inflammatory cytokines in LESC due to the examined conditions. Increased expression of *IL-1 α* and *IL-1 β* was observed in cells cultured in CM at standard conditions compared to cells with induced inflammation (CM_LPS). IL-1 α and IL-1 β role is associated with the regulation of an immune response as a result of ongoing infection [51, 52]. IL-1 also acts by activating the secretion of many different cytokines and chemokines, e.g. IL-6, TNF α and INF γ [51, 52]. IL-1 participate in the regulation of stem cell activity. In hematopoietic stem cells (HSCs), it exhibits radioprotective activity, induces their proliferation and differentiation [52]. Our findings showed no differences in the expression of *IL-1 α* and *IL-1 β* between cells cultured in CM and SM in both standard conditions and induced inflammation. The lower expression of both interleukins in CM_LPS group suggests that CM has a significant impact on IL-1 release during the inflammation. This activity was confirmed by ELISA detection of IL-1 α . The concentration of this IL-1 α in LESC'S culture medium was lower during induced inflammation under the influence of CM (CM_LPS group). Furthermore, significant difference in the secretion of IL-1 α between LECs with induced inflammation and LESC in standard conditions cultured in SM indicates that LPS has meaningful impact on IL-1 α release. Finally, observed no differences in IL-1 α secretion between CM, CM_LPS and SM group suggest that CM downregulates the release of IL-1 α which means that ADSC secrete factors which mitigates the inflammation. Some studies suggest that an increase in interleukin-1 secretion was observed under the influence of cellular stress e.g. in a state of hypoxia, chemical or physical damage. During the apoptosis which is natural, programmed cell death IL-1 α is not released by the cells [51]. Solomon et al. reported that stroma of amniotic membrane inhibits the level of IL-1 α and IL-1 β , after LPS stimulation, in cell culture of limbal epithelial stem cells [53]. It suggests that the use of CM simultaneously with amniotic membrane could give better therapeutic results in cornea treatment by achieving a synergistic effect. The analysis of the *IL-6* mRNA expression showed decreased copy number in cells with induced inflammation cultured in CM compared to SM. ELISA also indicated a significant decrease of IL-6 secretion by LESC with induced inflammation compared to cells cultured in SM. Similarly, lower concentration of IL-6 in the same group was noticed at LESC in standard conditions. Studies indicate that IL-6 secretion increase in cells during inflammation [54, 55]. However, it was proven that MSCs naturally secrete IL-6 [56, 57]. This interleukin is responsible for the regulation of the immune response, hematopoiesis, apoptosis, proliferation and cell viability [58]. It plays important role in regulation of homeostasis in corneal limbus cells niche. IL-6 may help in wound healing of epithelial cells *in vivo* [59, 60]. Perhaps a significant increase in the level of this interleukin may be the result of cellular responses to unfavorable environmental conditions e.g. like LPS induced inflammation. Lower levels of

IL-6 at LESC cultured in CM suggest a beneficial property of ADSC secreted factors. Probably factors present in the conditioned medium from ADSC promote the LESC regeneration. Perhaps the use of IL-6 as a supplement for cell culture could provide better proliferation. However, this issue requires further study. The analysis also revealed an increase of *IFN γ* mRNA expression in LESC with induced inflammation cultured in SM compared to the LECs in standard conditions cultured in the same medium. This indicates that LPS stimulation probably caused upregulation of this gene expression. However, IFN γ was not detected by ELISA. It should be remembered that the increase in mRNA level is ahead of the protein concentration. Gene expression depends on many transcription factors which activation changes the level of mRNA in cells. Thus, when translation product is at demanded level, the DNA transcription can be suppressed. In opposite, when there is a low level of protein, gene expression can be continuously upregulated.

Conclusion

Our results showed that the paracrine activity of ADSC on LESC promotes its proliferation and mitigates the adverse impact of LPS. We find the ADSC'S secreted factors useful for LESC regeneration. ADSC can serve as supporting cells for corneal wound healing. Placing the ADSC in closed carrier can probably be used for temporal wound dressing. The ADSC'S secretome could be used in development of new medicinal products for corneal injures.

Declarations

Acknowledgements

We would like to express our deepest appreciation to prof. dr hab. Urszula Mazurek and dr hab. Monika Paul-Samojedny for invaluable methodological support and sharing of laboratory facilities.

Authors kindly acknowledge the support from Medical University of Silesia in Katowice (Upper Silesia, Poland) grant no. KNW-1-032/N/9/I and PCN-2-004/N/0/O

Authors' contributions

B.S. and A.S-S. conceived and designed the experiments; B.S. and A.P. performed the experiments; A.S-S. and J.G. analyzed the data; J.G. contributed reagents/materials/analysis tools; B.S., A.S-S. wrote the paper.

Competing interests

The authors declare that there is no conflict of interest regarding the publication of this article

References

1. Willoughby, C. E. *et al.* Anatomy and Physiology of the Human Eye: Effects of Mucopolysaccharidoses Disease on Structure and Function - a Review: Anatomy and Physiology of the Eye. *Clin. Experiment. Ophthalmol.* **38**, 2–11 <https://doi.org/10.1111/j.1442-9071.2010.02363.x> (2010).
2. Le-Bel, G. *et al.* Grafting of an Autologous Tissue-Engineered Human Corneal Epithelium to a Patient with Limbal Stem Cell Deficiency (LSCD). *Am. J. Ophthalmol. Case Rep.* **15**, 100532 <https://doi.org/10.1016/j.ajoc.2019.100532> (2019).
3. Jankowska-Szmul, J. *et al.* Changes in Technique and Indications for Keratoplasty in Poland, 1989 to 2014: An Analysis of Corneal Transplantations Performed at Saint Barbara Hospital, Trauma Center, Sosnowiec, Poland. *Transplant. Proc.* **48**, 1818–1823, [doi:10.1016/j.transproceed.2016.01.056](https://doi.org/10.1016/j.transproceed.2016.01.056).
4. Lagali, N. *et al.* Stage-Related Central Corneal Epithelial Transformation in Congenital Aniridia-Associated Keratopathy. *Ocul. Surf.* **16**, 163–172 <https://doi.org/10.1016/j.jtos.2017.11.003> (2018).
5. Eye Bank Association of America Statistical Report Available online: <https://restoresight.org/what-we-do/publications/statistical-report/> (accessed on 29 August 2019).
6. Gain, P. *et al.* Global Survey of Corneal Transplantation and Eye Banking. *JAMA Ophthalmol.* **134**, 167 <https://doi.org/10.1001/jamaophthalmol.2015.4776> (2016).
7. Avadhanam, V. S. & Liu, C. S. C. A Brief Review of Boston Type-1 and Osteo-Odonto Keratoprotheses. *Br. J. Ophthalmol.* **99**, 878–887 <https://doi.org/10.1136/bjophthalmol-2014-305359> (2015).
8. Avadhanam, V. S., Smith, H. E. & Liu, C. Keratoprotheses for Corneal Blindness: A Review of Contemporary Devices. *Clin. Ophthalmol. Auckl. NZ.* **9**, 697–720 <https://doi.org/10.2147/OPHTH.S27083> (2015).
9. McCarthy, M. & Keratoprosthesis (eds) (Kpro) Available online <http://mccarthyeye.com/kpro/> (accessed on 30 June 2018).
10. Rafat, M., Hackett, J., Fagerholm, P. & Griffith, M. Artificial Cornea. In *Encyclopedia of the Eye*; Elsevier 2010; Vol. 1, pp. 128–134 ISBN 978-0-12-374203-2.
11. Choi, H. J. *et al.* Efficacy of Pig-to-Rhesus Lamellar Corneal Xenotransplantation. *Investig. Ophthalmology Vis. Sci.* **52**, 6643 <https://doi.org/10.1167/iovs.11-7273> (2011).
12. Hara, H., Cooper, D. K. C., XENOTRANSPLANTATION – THE & FUTURE OF CORNEAL TRANSPLANTATION?. *Cornea.* **30**, 371–378 <https://doi.org/10.1097/ICO.0b013e3181f237ef> (2011).
13. Wu, Z. *et al.* Reconstruction of Auto-Tissue-Engineered Lamellar Cornea by Dynamic Culture for Transplantation: A Rabbit Model. *PLOS ONE.* **9**, e93012 <https://doi.org/10.1371/journal.pone.0093012> (2014).
14. Jiang, Z. *et al.* Paracrine Effects of Mesenchymal Stem Cells on the Activation of Keratocytes. *Br. J. Ophthalmol.* [bjophthalmol https://doi.org/10.1136/bjophthalmol-2016-310012](https://doi.org/10.1136/bjophthalmol-2016-310012) (2017).
15. Ramaswamy, Y., Lim, K. S., Zreiqat, H. & Lu, Z. Stem Cells for Bone Regeneration: Role of Trophic Factors. In *Advanced Techniques in Bone Regeneration*; Zorzi, A.R., de Miranda, J.B., Eds.; InTech,

2016 ISBN 978-953-51-2538-9.

16. Liu, C. *et al.* Pretreatment of Mesenchymal Stem Cells with Angiotensin II Enhances Paracrine Effects, Angiogenesis, Gap Junction Formation and Therapeutic Efficacy for Myocardial Infarction. *Int. J. Cardiol.* **188**, 22–32 <https://doi.org/10.1016/j.ijcard.2015.03.425> (2015).
17. Zhou, Y., Yamamoto, Y., Xiao, Z. & Ochiya, T. The Immunomodulatory Functions of Mesenchymal Stromal/Stem Cells Mediated via Paracrine Activity. *J. Clin. Med.* **8**, 1025 <https://doi.org/10.3390/jcm8071025> (2019).
18. Hong, P., Yang, H., Wu, Y., Li, K. & Tang, Z. The Functions and Clinical Application Potential of Exosomes Derived from Adipose Mesenchymal Stem Cells: A Comprehensive Review. *Stem Cell Res. Ther.* **10**, 242 <https://doi.org/10.1186/s13287-019-1358-y> (2019).
19. Mendt, M., Rezvani, K. & Shpall, E. Mesenchymal Stem Cell-Derived Exosomes for Clinical Use. *Bone Marrow Transplant.* **54**, 789–792 <https://doi.org/10.1038/s41409-019-0616-z> (2019).
20. Wang, X. *et al.* Fetal Dermal Mesenchymal Stem Cell-Derived Exosomes Accelerate Cutaneous Wound Healing by Activating Notch Signaling. *Stem Cells Int.* 2019, 2019, 1–11, doi:10.1155/2019/2402916.
21. Lavoie, J. R. & Rosu-Myles, M. Uncovering the Secretes of Mesenchymal Stem Cells. *Biochimie.* **95** (12), 2212–2221 (2013).
22. Murphy, M. B., Moncivais, K. & Caplan, A. I. Mesenchymal Stem Cells: Environmentally Responsive Therapeutics for Regenerative Medicine. *Exp. Mol. Med.* **45**, e54 <https://doi.org/10.1038/emm.2013.94> (2013).
23. Shojaei, F., Rahmati, S. & Banitalebi Dehkordi, M. A. Review on Different Methods to Increase the Efficiency of Mesenchymal Stem Cell-based Wound Therapy. *Wound Repair Regen.* 2019, wr.12749, doi:10.1111/wrr.12749.
24. Yang, Y. H. *et al.* Stromal Tissue Rigidity Promotes Mesenchymal Stem Cell-Mediated Corneal Wound Healing Through the Transforming Growth Factor β Signaling Pathway: Stromal Rigidity Promotes MSC-Mediated Corneal Wound Healing via TGF- β . *STEM CELLS.* **34**, 2525–2535 <https://doi.org/10.1002/stem.2405> (2016).
25. Turani, M. *et al.* Antibiotics Delay in Vitro Human Stem Cell Regrowth. *Toxicol. In Vitro.* **29**, 370–379 <https://doi.org/10.1016/j.tiv.2014.10.013> (2015).
26. Dowling, P. & Clynes, M. Conditioned Media from Cell Lines: A Complementary Model to Clinical Specimens for the Discovery of Disease-Specific Biomarkers. *PROTEOMICS* 2011, **11**, 794–804, doi:10.1002/pmic.201000530.
27. Skubis, A. *et al.* Impact of Antibiotics on the Proliferation and Differentiation of Human Adipose-Derived Mesenchymal Stem Cells. *Int. J. Mol. Sci.* **18**, 2522 <https://doi.org/10.3390/ijms18122522> (2017).
28. Zajdel, A., Wilczok, A. & Tarkowski, M. Toxic Effects of N-3 Polyunsaturated Fatty Acids in Human Lung A549 Cells. *Toxicol. In Vitro.* **30**, 486–491 <https://doi.org/10.1016/j.tiv.2015.09.013> (2015).

29. Liang, C. C., Park, A. Y. & Guan, J. L. In Vitro Scratch Assay: A Convenient and Inexpensive Method for Analysis of Cell Migration in Vitro. *Nat. Protoc.* **2**, 329–333 <https://doi.org/10.1038/nprot.2007.30> (2007).
30. Bochner, B. R. *et al.* Assay of the Multiple Energy-Producing Pathways of Mammalian Cells. *PLoS ONE*. **6**, e18147 <https://doi.org/10.1371/journal.pone.0018147> (2011).
31. Gola, J. *et al.* –Copper (II) Complex Alters Transcriptional Activity of Genes Encoding Transforming Growth Factor-Beta Family Members and Related Proteins in Renal Cells. *Pharmacol. Rep.* **69**, 1308–1314 <https://doi.org/10.1016/j.pharep.2017.05.011> (2017).
32. Kukolj, T. *et al.* Lipopolysaccharide Can Modify Differentiation and Immunomodulatory Potential of Periodontal Ligament Stem Cells via ERK1,2 Signaling. *J. Cell. Physiol.* **233**, 447–462 <https://doi.org/10.1002/jcp.25904> (2018).
33. Shi, H. *et al.* The in Vitro Effect of Lipopolysaccharide on Proliferation, Inflammatory Factors and Antioxidant Enzyme Activity in Bovine Mammary Epithelial Cells. *Anim. Nutr.* **2**, 99–104 <https://doi.org/10.1016/j.aninu.2016.03.005> (2016).
34. Yang, H., Hu, C., Li, F., Liang, L. & Liu, L. Effect of Lipopolysaccharide on the Biological Characteristics of Human Skin Fibroblasts and Hypertrophic Scar Tissue Formation. *IUBMB Life*. **65**, 526–532 <https://doi.org/10.1002/iub.1159> (2013).
35. Wang, J. *et al.* Dynamic Changes of Phenotypes and Secretory Functions during the Differentiation of Pre-DCs to Mature DCs. *J. Huazhong Univ. Sci. Technolog. Med. Sci.* **37**, 191–196 <https://doi.org/10.1007/s11596-017-1714-z> (2017).
36. Chanrot, M. *et al.* Dose Related Effects of LPS on Endometrial Epithelial Cell Populations from Dioestrus Cows. *Anim. Reprod. Sci.* **177**, 12–24 <https://doi.org/10.1016/j.anireprosci.2016.12.002> (2017).
37. Chen, X., Zhang, Z. Y., Zhou, H. & Zhou, G. W. Characterization of Mesenchymal Stem Cells under the Stimulation of Toll-like Receptor Agonists. *Dev. Growth Differ.* **56**, 233–244 <https://doi.org/10.1111/dgd.12124> (2014).
38. Kimsa, M. *et al.* Transforming Growth Factor β -Related Genes in Human Retinal Pigment Epithelial Cells after Tacrolimus Treatment. *Pharmacol. Rep.* **68**, 969–974 <https://doi.org/10.1016/j.pharep.2016.04.020> (2016).
39. Nakajima, V. M. *et al.* Biotransformed Citrus Extract as a Source of Anti-Inflammatory Polyphenols: Effects in Macrophages and Adipocytes. *Food Res. Int.* **97**, 37–44 <https://doi.org/10.1016/j.foodres.2017.03.034> (2017).
40. Chen, J. *et al.* Mesenchymal Stem Cell Conditioned Medium Promotes Proliferation and Migration of Alveolar Epithelial Cells under Septic Conditions In Vitro via the JNK-P38 Signaling Pathway. *Cell. Physiol. Biochem.* **37**, 1830–1846 <https://doi.org/10.1159/000438545> (2015).
41. Farahmand, L., Esmaeili, R., Eini, L. & Majidzadeh-A, K. The Effect of Mesenchymal Stem Cell-Conditioned Medium on Proliferation and Apoptosis of Breast Cancer Cell Line. *J. Cancer Res. Ther.* **14**, 341 (2018).

42. Kay, A. G. *et al.* Mesenchymal Stem Cell-Conditioned Medium Reduces Disease Severity and Immune Responses in Inflammatory Arthritis. *Sci. Rep.* **7**, <https://doi.org/10.1038/s41598-017-18144-w> (2017).
43. Li, M. *et al.* Mesenchymal Stem Cell-Conditioned Medium Improves the Proliferation and Migration of Keratinocytes in a Diabetes-Like Microenvironment. *Int. J. Low. Extrem. Wounds.* **14**, 73–86 (2015).
44. Waszak, P. *et al.* Preconditioning Enhances the Paracrine Effect of Mesenchymal Stem Cells in Preventing Oxygen-Induced Neonatal Lung Injury in Rats. *Stem Cells Dev.* **21**, 2789–2797 <https://doi.org/10.1089/scd.2010.0566> (2012).
45. Zhang, Q., Liu, L. N., Yong, Q., Deng, J. C. & Cao, W. G. Intralesional Injection of Adipose-Derived Stem Cells Reduces Hypertrophic Scarring in a Rabbit Ear Model. *Stem Cell Res. Ther.* **6**, 145 <https://doi.org/10.1186/s13287-015-0133-y> (2015).
46. Zeppieri, M. *et al.* Human Adipose-Derived Stem Cells for the Treatment of Chemically Burned Rat Cornea: Preliminary Results. *Curr. Eye Res.* **38**, 451–463 <https://doi.org/10.3109/02713683.2012.763100> (2013).
47. Galindo, S. *et al.* Therapeutic Effect of Human Adipose Tissue-Derived Mesenchymal Stem Cells in Experimental Corneal Failure Due to Limbal Stem Cell Niche Damage. *Stem Cells Dayt. Ohio.* **35**, 2160–2174 <https://doi.org/10.1002/stem.2672> (2017).
48. Huh, J. E. & Lee, S. Y. IL-6 Is Produced by Adipose-Derived Stromal Cells and Promotes Osteogenesis. *Biochim. Biophys. Acta BBA - Mol. Cell Res.* **1833**, 2608–2616 <https://doi.org/10.1016/j.bbamcr.2013.06.025> (2013).
49. Larocca, R. A. *et al.* Adipose Tissue-Derived Mesenchymal Stem Cells Increase Skin Allograft Survival and Inhibit Th-17 Immune Response. *PLoS ONE.* **8**, e76396 <https://doi.org/10.1371/journal.pone.0076396> (2013).
50. Wei, H. J. *et al.* Adipose-Derived Stem Cells Promote Tumor Initiation and Accelerate Tumor Growth by Interleukin-6 Production. *Oncotarget* 2015, **6**, 7713.
51. Lukens, J. R., Gross, J. M. & Kanneganti, T. D. IL-1 Family Cytokines Trigger Sterile Inflammatory Disease. *Front. Immunol.* **3**, <https://doi.org/10.3389/fimmu.2012.00315> (2012).
52. Orelio, C., Haak, E., Peeters, M. & Dzierzak, E. Interleukin-1-Mediated Hematopoietic Cell Regulation in the Aorta-Gonad-Mesonephros Region of the Mouse Embryo. *Blood.* **112**, 4895–4904 <https://doi.org/10.1182/blood-2007-12-123836> (2008).
53. Solomon, A. *et al.* Suppression of Interleukin 1 α and Interleukin 1 β in Human Limbal Epithelial Cells Cultured on the Amniotic Membrane Stromal Matrix. *Br. J. Ophthalmol.* **85**, 444–449 <https://doi.org/10.1136/bjo.85.4.444> (2001).
54. Beurel, E., Jope, R. S. & Lipopolysaccharide-Induced Interleukin-6 Production Is Controlled by Glycogen Synthase Kinase-3 and STAT3 in the Brain. *J. Neuroinflammation.* **6**, <https://doi.org/10.1186/1742-2094-6-9> (2009).

55. Eggesbø, J. B., Hjermann, I., Høstmark, A. T. & Kierulf, P. L. P. S. Induced Release of IL-1 β , IL-6, IL-8 and TNF- α in EDTA or Heparin Anticoagulated Whole Blood from Persons with High or Low Levels of Serum HDL. *Cytokine*. **8**, 152–160 <https://doi.org/10.1006/cyto.1996.0022> (1996).
56. Mi, F. & Gong, L. Secretion of Interleukin-6 by Bone Marrow Mesenchymal Stem Cells Promotes Metastasis in Hepatocellular Carcinoma. *Biosci. Rep.* **37**, BSR20170181 <https://doi.org/10.1042/BSR20170181> (2017).
57. Wu, Y. *et al.* Adipose Tissue-Derived Mesenchymal Stem Cells Have a Heterogenic Cytokine Secretion Profile. *Stem Cells Int.* 2017, *2017*, doi:10.1155/2017/4960831.
58. Kyurkchiev, D. *et al.* Secretion of Immunoregulatory Cytokines by Mesenchymal Stem Cells. *World J. Stem Cells*. **6**, 552–570 <https://doi.org/10.4252/wjsc.v6.i5.552> (2014).
59. Notara, M., Shortt, A. J., Galatowicz, G., Calder, V. & Daniels, J. T. IL6 and the Human Limbal Stem Cell Niche: A Mediator of Epithelial–Stromal Interaction. *Stem Cell Res.* **5**, 188–200 <https://doi.org/10.1016/j.scr.2010.07.002> (2010).
60. Veréb, Z. *et al.* Comparison of Upstream Regulators in Human Ex Vivo Cultured Cornea Limbal Epithelial Stem Cells and Differentiated Corneal Epithelial Cells. *BMC Genomics*. **14**, 900 (2013).

Figures

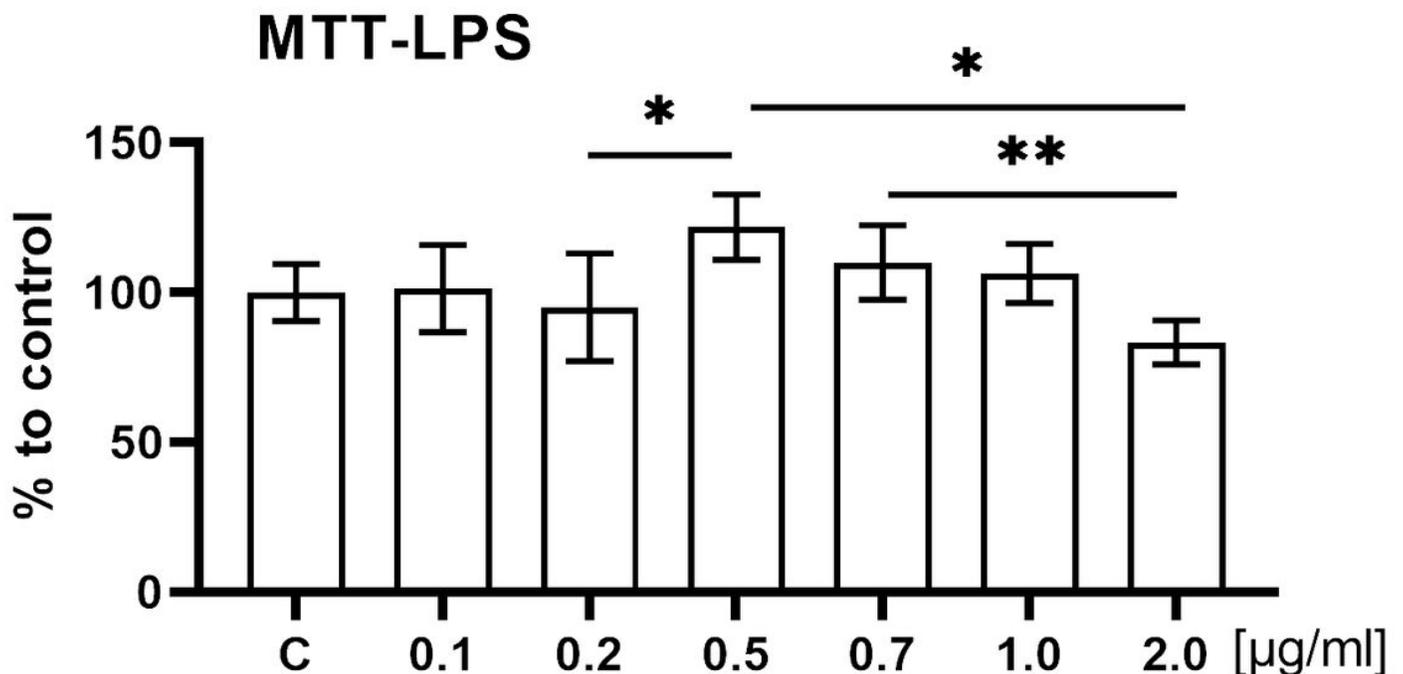


Figure 1

Cells viability based on the measurement of mitochondrial oxidative activity with MTT assay after exposure of cells to LPS. The bars represent the means \pm standard deviation (SD) of the percentages of

the control cell viability (100%); ANOVA with the Tukey post hoc test, * $p < 0.05$, ** $p < 0.01$.

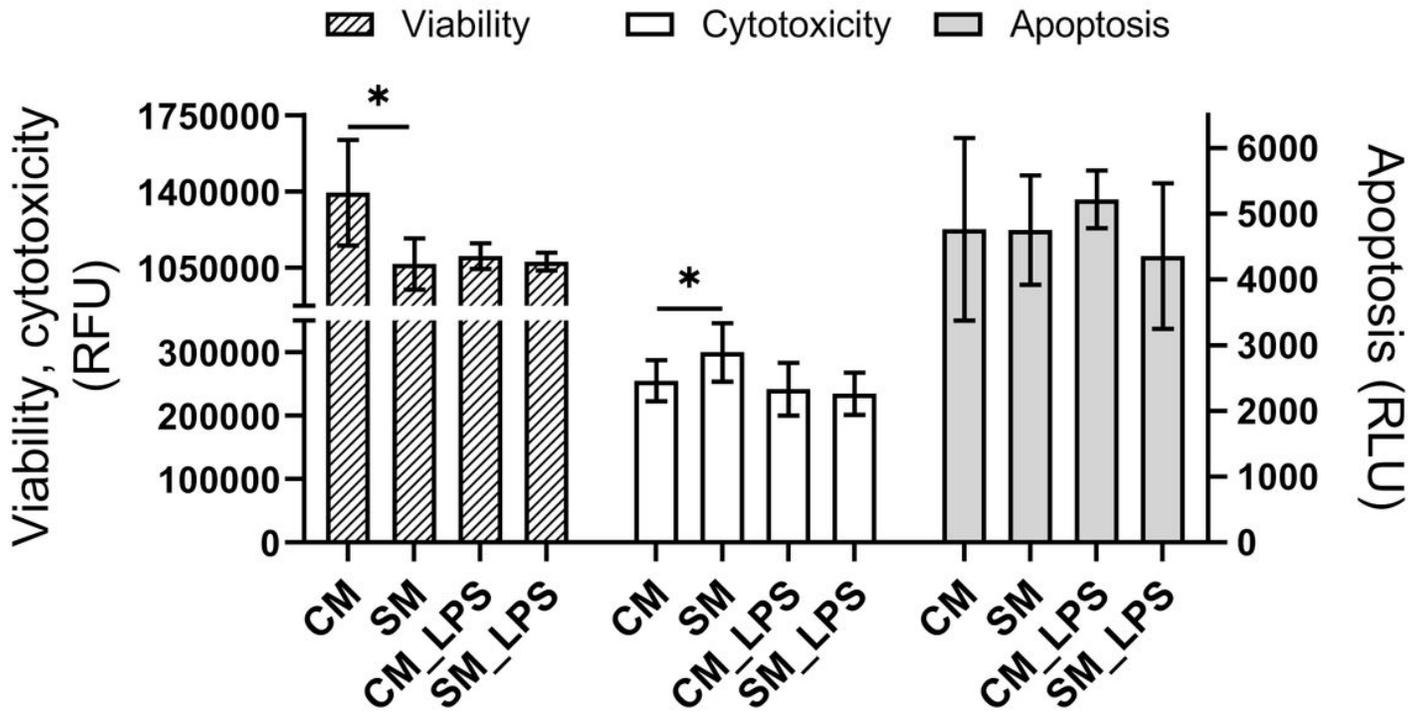


Figure 2

Cells viability, cytotoxicity and apoptosis of CM, SM, CM_LPS, SM_LPS groups. The activity of the dead-cell proteases (viability and cytotoxicity) and the activity of caspase 3/7 (apoptosis) are shown as relative fluorescence units or the luminescence intensity. The bars represent the means \pm standard deviation (SD). T test, * $p < 0.05$.

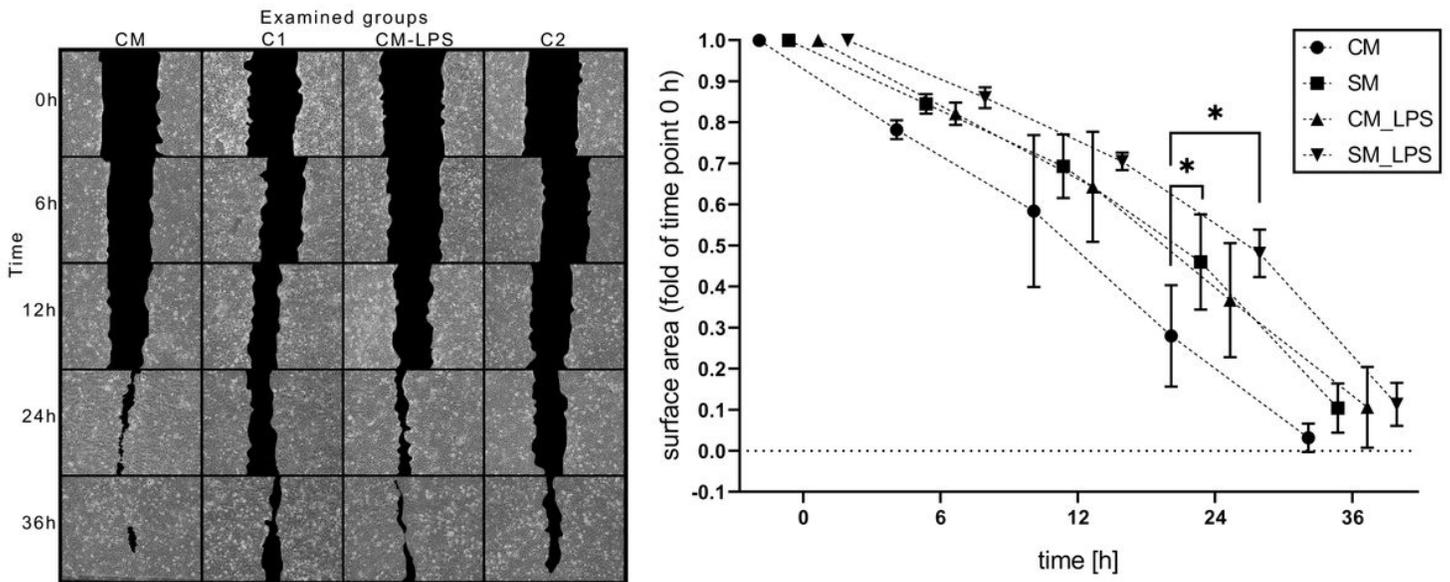


Figure 3

Left: A. Scratch assay. The results present a microscopic image of the selected repetition for each group (CM, SM, CM_LPS, SM_LPS) at time points (0h, 6h, 12h, 24h, 36h). Scratch area marked with black. C1 = SM, C2 = SM_LPS Right: B. Scratch surface area as fold of time point 0h of examined groups (CM, SM, CM_LPS, SM_LPS) in time (6h, 12h, 24h, 36h). T test, *p<0.05.

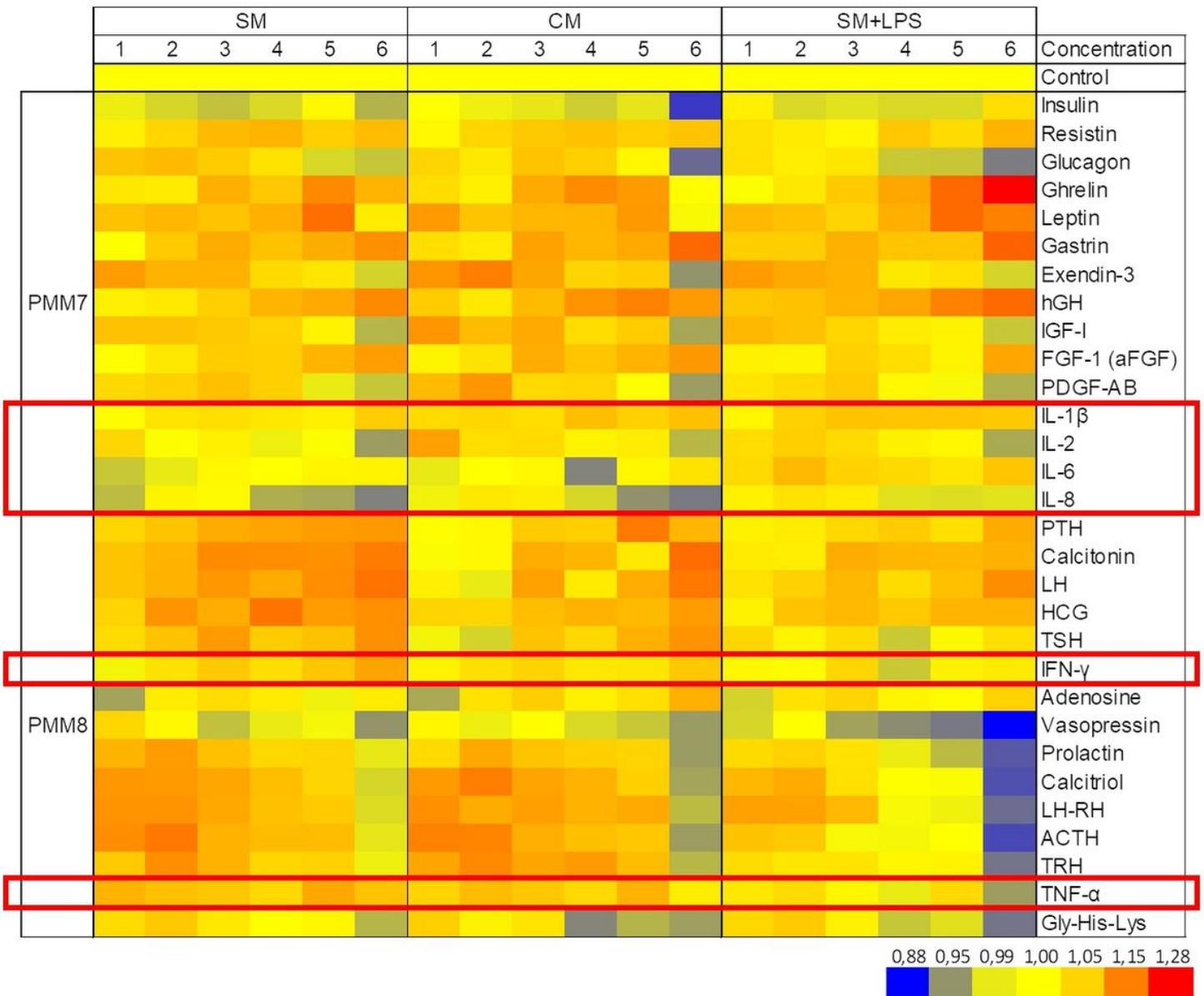


Figure 4

Heatmap of fold change in Phenotype MicroArrays for mammalian cells, PMM7 and PMM8 – „MicroPlate™ - Hormones & Metabolic Effectors“ in examined groups. SM - standard medium, CM - conditioned medium, SM+LPS – standard medium with the addition of 2 μ g/ml LPS. Red – highest values, blue – lowest values, yellow – neutral. Interleukins are marked with red frame.

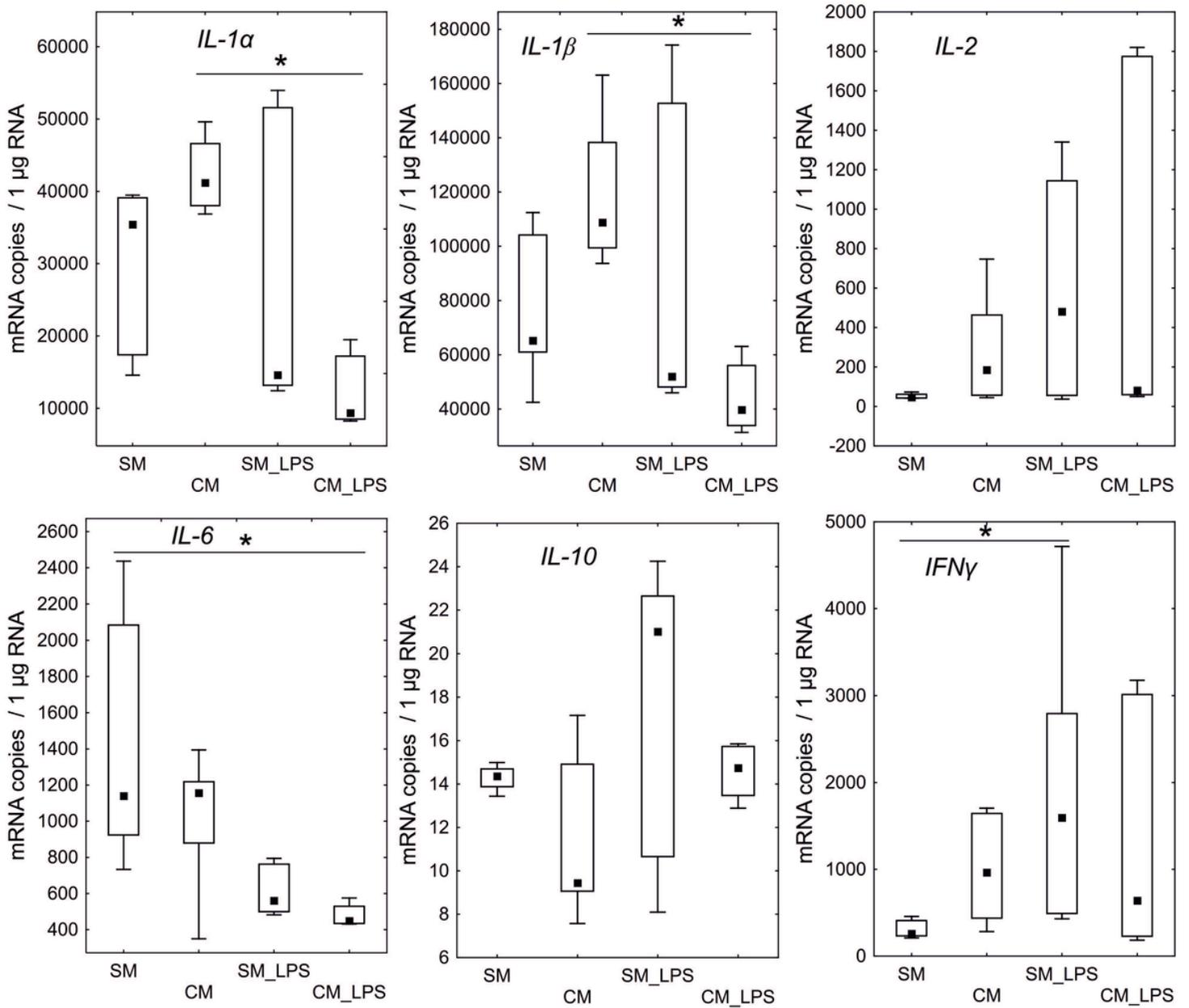


Figure 5

The mRNA levels of inflammation markers IL-1 α , IL-1 β , IL-2, IL-6, IL-10, IFN γ . The bars represent the (Me) with the 25th and 75th quartiles and the minimum and maximum of the copy numbers per 1 μ g of total RNA. The Kruskal Wallis test with post hoc was applied to assess any differences in the expression of the genes, *p<0,05.

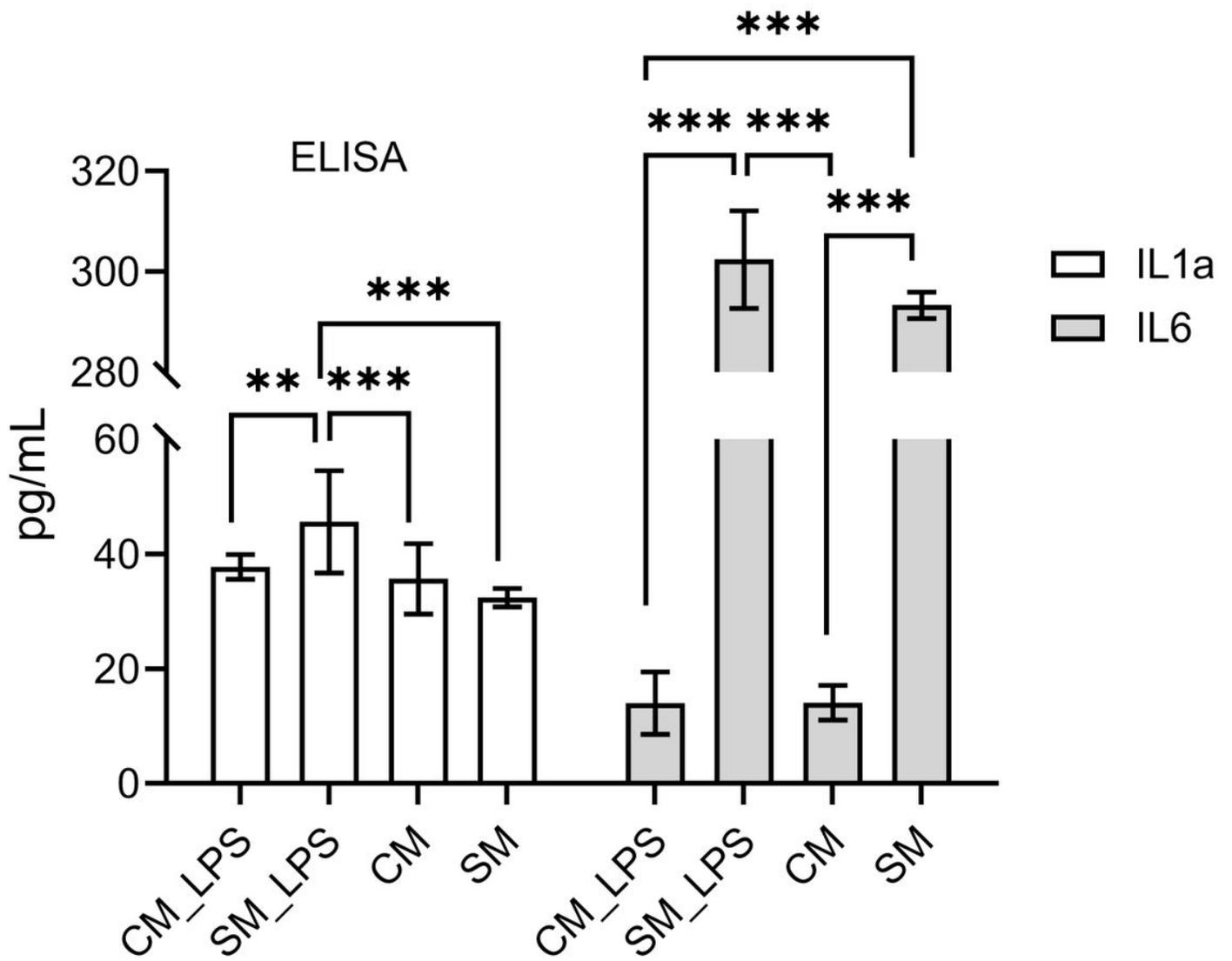


Figure 6

Secretion of cytokines by LESC in all examined groups (CM, SM, CM_LPS, SM_LPS) measured by ELISA. ANOVA with the Tukey post hoc test; IL6: **p<0,01, ***p<0,001