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Suppression of T cells by mesenchymal and cardiac progenitor cells is partly mediated via extracellular vesicles

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Abstract

Adverse remodeling after myocardial infarction (MI) is strongly influenced by T cells. Stem cell therapy after MI, using mesenchymal stem cells (MSC) or cardiomyocyte progenitor cells (CMPC), improved cardiac function, despite low cell retention and limited differentiation. As MSC secrete many factors affecting T cell proliferation and function, we hypothesized the immune response could be affected as one of the targets of stem cell therapy. Therefore, we studied the immunosuppressive properties of human BM-MSC and CMPC and their extracellular vesicles (EVs) in co-culture with activated T cells. Proliferation of T cells, measured by carboxyfluorescein succinimidyl ester dilution, was significantly reduced in the presence of BM-MSC and CMPC. The inflammatory cytokine panel of the T cells in co-culture, measured by Luminex assay, changed, with strong downregulation of IFN-gamma and TNF-alpha. The effect on proliferation was observed in both direct cell contact and transwell co-culture

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systems. Transfer of conditioned medium to unrelated T cells abrogated proliferation in these cells. EVs isolated from the conditioned medium of BM-MSC and CMPC prevented T cell proliferation in a dose-dependent fashion. Progenitor cells presence induces up- and downregulation of multiple previously unreported pathways in T cells. In conclusion, both BM-MSC and CMPC have a strong capacity for *in vitro* immunosuppression. This effect is mediated by paracrine factors, such as extracellular vesicles. Besides proliferation, many additional pathways are influenced by both BM-MSC and CMPC.

Keywords: Immunology, Stem cell research

1. Introduction

In Europe ischemic heart disease remains the most common cause of death, responsible for the death of 19% of males and 20% of females [1]. After ischemia and the subsequent reperfusion, a strong immune response ensues [2, 3, 4]. Many types of circulating immune cells, such as neutrophils, monocytes, and dendritic cells are recruited to the heart [4, 5, 6, 7]. Some of these cells pick up cardiac actin and myosin from the post-infarct debris and present them to T cells, which can then become auto-reactive for these cardiac antigens [6, 8, 9, 10]. The auto-reactive T cells can attack cardiac cells displaying these antigens for a long time after the initial event, leading to adverse remodeling and a gradually decreasing heart function [11, 12]. In fact, experimental models show the transfer of immune cells, including T cells, from donors with cardiac disease will lead to decreased heart function in healthy recipients [12, 13]. Currently, none of the heart failure therapies are aimed at modulating this inflammatory process. The available immunosuppressive drugs suppress indiscriminately and can cause severe side effects, such as cardiac rupture [14, 15, 16].

Recently a lot of focus has been on the use of stem cell therapy to regenerate the damaged heart. Studies using different kind of progenitor cells, such as mesenchymal stem cells (MSC) or cardiomyocyte progenitor cells (CMPC), show a reduction in the decrease of cardiac function after MI [17, 18, 19, 20, 21], which is maintained up to at least three months after cell administration [17]. However, poor engraftment has been observed for both MSC and CPMC [17, 18, 19, 22] and, in the case of MSC, cardiac-differentiated cells have rarely been found [18]. This strengthens the hypothesis that most beneficial effects of cardiac stem cell injections arise from the secretion of paracrine factors [17, 22, 23]. Paracrine factors produced by stem cells can direct many processes, including stimulation of cardiomyocyte survival and angiogenesis, which could improve outcome after MI [18, 22]. An often overlooked area of interest could be cardiac inflammation, as MSC are well known for their immunomodulating actions [14, 24, 25, 26]. MSC are able to reduce inflammation by suppressing the different cells of the immune system or force them

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into anti-inflammatory or even regulatory subtypes [14, 25, 26]. In addition, MSC are known to secrete extracellular vesicles (EVs) [27, 28], which are small lipid bi-layered vesicles with a diameter between 30-100nm, containing a specific mixture of proteins, peptides, lipids and genetic material [28]. These EVs function as a form of intracellular communication and are able to influence many processes in the body, including inflammation [21, 27, 28, 29, 30]. For this reason, we compared the effect both BM-MSC, CMPC and their EVs have on the activated immune system and in specific, how they alter allogeneic T cell responses *in vitro*.

2. Materials and methods

2.1. Cell culture

After approval of the Ethics Committee and obtaining informed consent from the donors, human bone marrow-derived BM-MSC and human CMPC, both adult and fetal, were obtained and characterized in our lab as described previously [23, 31, 32]. The adult, patient-derived progenitor cells were obtained from patients with severe heart failure due to ischemia. Of both BM-MSC and CMPC four different donors were used between passage 6 and passage 17. Cells were cultured in plastic culture flasks coated with 0.1% gelatin. BM-MSC were cultured in MEM-alpha (Gibco, 22561) supplemented with 10% fetal bovine serum (FBS; Gibco, 10099-141), 100U/ml Penicillin and 100 μg/ml Streptomycin (Lonza, 17-602E), 1 ng/ml bFGF (Sigma F0291) and 0.2 mM L-ascorbic acid-2-phosphate (Sigma A4034), as described before [19]. CMPC were cultured in CMPC culture medium (1 part endothelial basal medium (EGM-2; Lonza CC-3156) and 3 parts M199 (Lonza BE12-119F) supplemented with 10% FBS, 100 U/ml Penicillin and 100 μg/ml Streptomycin and 1% Non-essential Amino Acids (Lonza 13-114), as described before [31, 33]. Both BM-MSC and CMPC were passaged when reaching 80−90% confluence by trypsin digestion (0.25% Trypsin; Lonza, CC-5012) at 37 °C for maximally two minutes.

Endothelial colony forming cells (ECFCs) were isolated from human umbilical cord blood and subsequently characterized as previously described [34]. Briefly, the mononuclear cell (MNC) fraction was isolated from whole blood using Ficoll-paque density gradient centrifugation (GE life sciences, 17-1440-02). MNCs were plated on rat-tail collagen type I (BD Biosciences, 354236) coated six-well culture plates in a final concentration of 2×10^7 cells per well in endothelial growth medium, consisting of EGM-2 supplemented with 10% FBS, 1% GlutaMax (Gibco, 35050038), 100U/ml Penicillin and 100 μg/ml Streptomycin. Medium was refreshed daily for the first four days. After day seven the cells were trypsinized and plated on fresh collagen type I coated wells until colonies appeared. ECFC colonies were isolated and passaged at 90% confluency.

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To generate conditioned medium (CM), cell cultures were maintained at 37 °C for at least 3 days in a humidified atmosphere (5% CO_2 and 20% O_2). The CM was directly used in experiments, used for isolation of EVs, or stored in −20 °C for later use.

2.2. T cell isolation

Human blood of healthy volunteers was collected via the in-hospital donor-service after obtaining written consent. This donor service has been approved by the Ethics Committee of the University Medical Centre Utrecht and complies with the Declaration of Helsinki and the Good Clinical Practice guidelines. T cells were freshly isolated from the blood using the subsequent steps: Peripheral blood mononuclear cells (PBMC) were purified by a Ficoll-Paque density gradient (1.077 g/mL, GE healthcare, 17-1440-02), according to the manufacturer's protocol [23, 35]. Using anti-CD3 magnetic beads (BD Biosciences, 552593) and the BD IMagnet (BD Bioscience, 552311), T cells were isolated from the PBMCs according to manufacturer's protocol. T cells were labeled with 1.5μM carboxyfluorescein succinimidyl ester (CFSE; Sigma, 21888), as described previously [23]. CFSE was diluted stepwise to the desired concentration and incubated with the cells for 10 minutes at 37 °C in a dark, shaking water bath. Afterwards 5% FBS was added to block further uptake and cells were washed twice to remove excess CFSE.

2.3. Proliferation assay

T cell proliferation was determined in the presence of BM-MSC, CMPC, their conditioned medium or EVs. For this, stem cells were plated at a concentration of $5.0*10^4$ cells per well in a 48-wells plate. In case of the pre-conditioning experiments, 20 ng/mL IFN-gamma (Sigma I3265) was added. After 24 hours, the medium was removed and $5.0*10^4$ freshly isolated, CFSE-labeled T cells were added in RPMI-1640 (Lonza, BE12-702F) supplemented with 10% autologous human serum, 100U/ml Penicillin and 100 μg/ml Streptomycin.

T cells were activated using phorbol 12-myristate 13-acetate (PMA 0.123 ng/ml; Sigma, P8139) and IL-2 (120 IU/ml, BD Pharmingen, 554563) as described before [23]. After a six-day culture, cells were collected and Sytox blue (1μM; Invitrogen, S34857) was added to determine viability. Cell viability and proliferation was measured by flow cytometry (Gallios, Beckmann Coulter) and analyzed using Kaluza Analysis Software (Beckman Coulter, version 1.3) as followed: The fluorescent CFSE signal upon each cell division allowed us to count the number of cells present in each division. From this, we could calculate what percentage of the initial population had divided at the time of measurement (Suppl. Fig. 1). To allow comparisons between different donors, proliferation of the stimulated T cell sample was used for normalization. Where indicated 1-MT (Sigma 447439), Indomethacin (Sigma I7378) and Alk5 (Sigma S4696) were added at the start of the co-culture.

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2.4. Cytokine analysis

Conditioned medium (CM) was collected from CMPC, BM-MSC and T cells or from CMPC and BM-MSC co-culture with T cells after 6 days. Conditioned medium was centrifuged at 500× g for 10 minutes and supernatant was collected. After filtering through a 0.2 μm filter (Corning, 431219), the CM was stored in −20 °C for analysis and further experiments.

Cytokines were measured using the multiplex immunoassay system (BioPlex, 200; Bio-Rad Laboratories) combined with the BioPlex Precision Pro Human Cytokine 10-Plex Panel (Bio Rad 171-A1001P), according to the manufacturer's protocol. This multiplex assay detects IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12 (p70), IL-13, IFN-gamma and TNF-alpha. For analysis, the data was normalized to stimulated T cells to allow optimal comparisons between the different donors and groups.

2.5. Conditioned medium experiments

In the experiments where CM was used for T cell suppression, $2.0*10^4$ freshly isolated, CFSE-labeled T cells were added per well in a 96 wells plate and CM was added 1:1 with fresh medium. T cells were collected for analysis by flow cytometry after 4 days of culture.

2.6. Extracellular vesicle (EV) isolation and purification

For the isolation of EVs, CMPC and BM-MSC were cultured in EV-free medium. Hereto all serum components were centrifuged 60 minutes at 150,000 x g. EVs were isolated and validated from the CM by differential centrifugation as described before [36]. The CM was subsequently centrifuged for 15−30 minutes at 2,000 g, for 30 minutes at 10,000 g at 4 °C (Beckman, Optima LE-80K Ultracentrifuge), and finally pelleted in a final centrifugation step (60 minutes at 100,000 g at 4 °C). The EV pellet was washed with PBS and pelleted by another centrifugation step for 60 minutes at 100,000 g at 4 °C. Finally, the washed pellets were resuspended in PBS and before their use in functional experiments total EV protein concentration was determined with BCA protein assay (ThermoScientific). Further characterization of the obtained EVs in our lab was performed as described previously [27, 36, 37]. To determine the effect of CMPC and BM-MSC derived EVs on activated T cells, these exosomes were added immediately after PMA and IL-2 activation. After six days, the cells were collected and analyzed by flow cytometry. All functional tests were performed with unlabeled EVs.

To visualize uptake, the EV pellet was stained with PKH-26 (Sigma, PKH26GL), and after labeling excess PKH-26 was inactivated with EV-free FBS. PKH-26-labeled EVs and excess PKH-26 were separated by sucrose gradient purification and subsequently pelleted again by centrifugation at 100,000 g. The labeled EVs were added to CFSE-labeled T cells. After incubation of various durations, T cells were trapped on glass

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slides, the nucleus was stained with Hoechst 1:10,000 (Invitrogen H3570), fixed with 4% paraformaldehyde, and analyzed with fluorescence microscopy.

2.7. RNA sequencing

Samples for the RNAseq were isolated and put in co-culture as described above. We included T cells from three different donors and progenitor cells from two different donors. After 3 days, the non-adherent T cells were collected, washed twice with PBS and stored at −80 °C. In the end, 16 samples were selected based on quality, including both unstimulated (n = 3) and stimulated (n = 3) T cells, as well as stimulated T cells which had been in contact with BM-MSC (n = 5) or CMPC (n = 5). RNA was isolated and libraries were created using the TruSeq Stranded Total RNA Sample Preparation LS according to manufacturer's protocol. An Illumina NextSeq500 and read-count analysis was performed by the Utrecht DNA Sequencing Facility (Utrecht, the Netherlands). RNA-seq reads were aligned to the human reference genome GRCh37 using STAR version2.4.2a [http://bioinformatics.oxfordjournals.org/content/29/1/15]. Read groups were added to the BAM files with Picard's AddOrReplaceReadGroups (v1.98). The BAM files are sorted with Sambamba v0.4.5 and transcript abundances are quantified with HTSeq-count version 0.6.1p1 [https://doi.org/10.1093/bioinformatics/btu638] using the union mode. Subsequently, RPKM's are calculated with edgeR's rpkm() function [https://doi.org/10.1093%2Fbioinformatics%2Fbtp616].

The resulting read counts per mRNA were subsequently analyzed according to the DESeq2 pipeline, to calculate differential expression (padj < 0.05) between the 4 different groups of samples [38]. PCA analysis was performed using DESeq2 command plotPCA() with "ntop = 5000" parameter.

2.8. Data analysis

All data are reported as mean ± SEM. Analysis was performed with IBM SPSS 20.0. For group comparison, parametric (one-way ANOVA) or non-parametric (Kruskal-Wallis) analysis was performed followed by a LSD and Mann-Whitney post-hoc analysis with a Bonferroni correction for significance respectively. A p-value <0.05 was considered significant.

3. Results

3.1. BM-MSC and CMPC suppress proliferation of allogeneic T cells

Several forms of T-cell stimulation were tested, including IL-2/PHA, CD3/CD28 beads, and mixed lymphocyte reactions, yet we decided to work with IL-2/PMA

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as it gave the strongest proliferative response. Upon stimulation T cells form proliferation clusters (Fig. 1A). In the presence of BM-MSC and CMPC this cluster formation was strongly reduced or even absent, whereas in the presence of endothelial colony forming cells (ECFC) proliferating T cell colonies still formed (Fig. 2). After checking for viability, proliferation of the T cells was measured and subsequently calculated based on CFSE-signal intensity using flow cytometry (Fig. 1B+C). No differences in viability were observed between the groups. In the non-stimulated conditions no proliferation could be observed, indicating the isolation process itself did not activate the T cells. In IL-2/PMA-stimulated cultures, the percentage of proliferating T cells was significantly lower in the presence of BM-MSC (65% ± 8%;

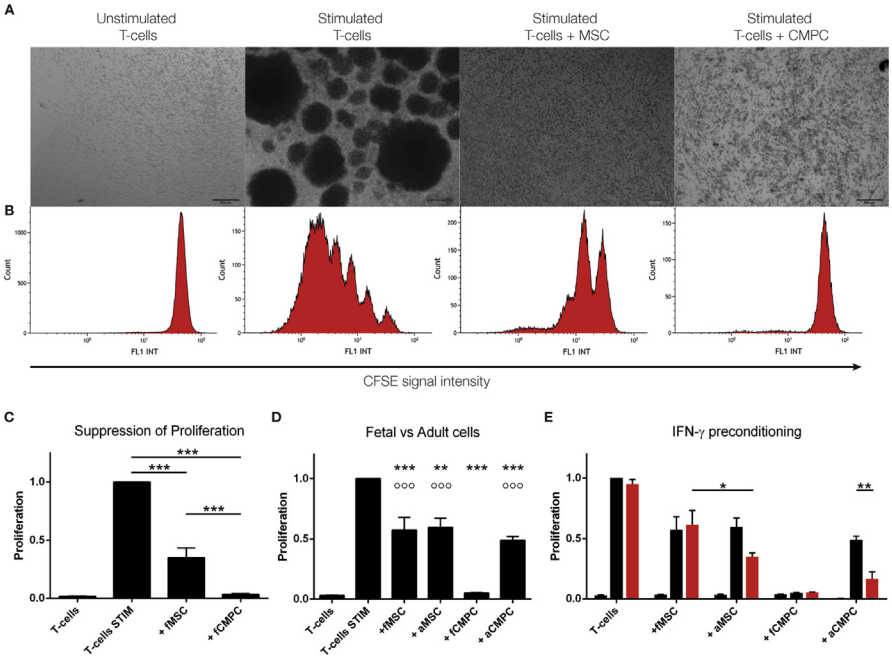

Fig. 1. BM-MSC and CMPC co-cultures with T cells after 6 days of stimulation. A. Light microscopical representation of T cell co-cultures after 6 days. In the non-stimulated samples, small individual cells are spread throughout the well. Upon stimulation, T cells form proliferating colonies. In the presence of BM-MSC and CMPC, the formation of these colonies is strongly reduced or even absent. Bar is 200 μm. B. Proliferation of T cells as measured by flow cytometry. Non-stimulated T cells have a single FL1 peak at a high fluorescent intensity. Upon stimulation, lower intensity peaks form, halving the fluorescent signal upon each cell division. C. Quantification of proliferation of T cells in different co-cultures. The first bar represents the unstimulated control, while the second bar is the stimulated T cells. Proliferation of T cells is significantly reduced in the presence of BM-MSC (65% ± 8) and CMPC (97% ± 0.6) (BM-MSC and CMPC: n = 12). D. Differences in suppressive capacity based on donor age. Both fetal and adult cells suppress T cell proliferation significantly. Adult BM-MSC suppress similar to fetal BM-MSC (40% ± 7.5 and 43% ± 11, resp.), while adults CMPC perform worse than fetal CMPC (51% ± 3.1 and 95% ± 0.4, resp.). E. The additional effect on T cell suppression due to 'licensing' was investigated. Progenitor cells were preconditioned with 20 ng/mL IFN-gamma (red bars) and compared to the unconditioned cells (black bars). Preconditioning had no effect on fetal stem cells. Adult BM-MSC improved to 65% ± 3 suppression, while adult CMPC improved to 83% ± 5.8 suppression ($p = 0.006$). ***$p < 0.001$ and **$p < 0.01$ compared to stimulated T cells. °°°$p < 0.001$ compared to T cells + fCMPC.

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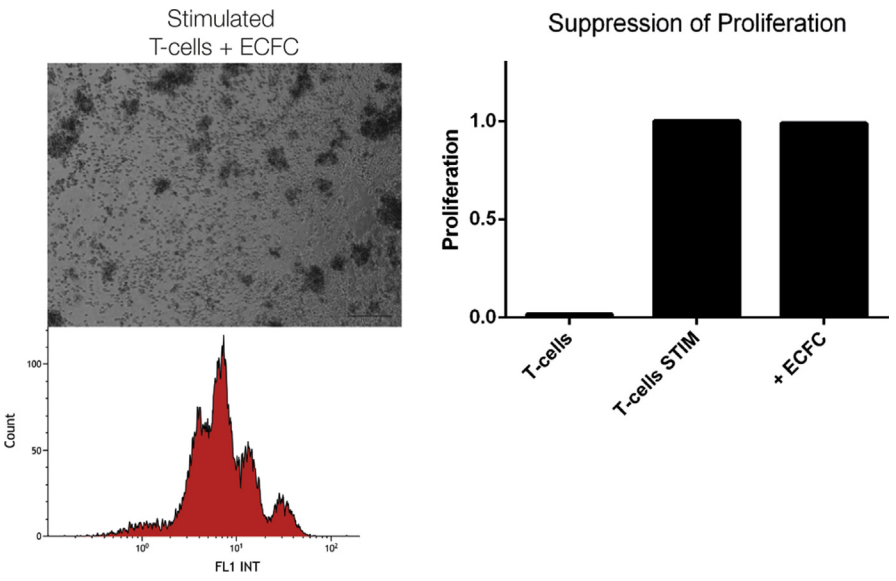

Fig. 2. ECFC do not suppress number or proliferating T cells. A similar co-culture was performed with several endothelial colony forming cell (ECFC) donors. Unlike the mesenchymal stem cell and the cardiomyocyte progenitor cell, the ECFC were not able to prevent the formation of the T cell clusters or prevent proliferation (0 ± 0.2 suppression).

$p < 0.001$) or CMPC ($97\% \pm 0.6\%$; $p < 0.001$; Fig. 1C). ECFC, on the other hand, did not influence the proliferation of T cells (Fig. 2; $0\% \pm 0.2\%$). Comparing the two types of progenitor cells, the CMPC perform significantly better than the BM-MSC (Fig. 1C; $p < 0.001$). As there evidence that the age or health of the donor can affect the function of stem cells [39, 40], we tested whether there is a difference in potency when comparing the fetal-derived progenitor to adult, patient-derived progenitor cells (Fig. 1D). With respect to the BM-MSC, the age or health status of the donor did not impact the suppressive capacities of the cells. This is not the case for the CMPC, where the fetal CMPC performed significantly better than the diseased adult CMPC ($p < 0.001$). Even so, the adult CMPC still maintain immunosuppressive abilities comparable to fetal and adult BM-MSC.

Several publications have shown that progenitor cells need to be 'licensed' before they show any suppressive capacities [41, 42, 43]. We investigated whether preconditioning affected the suppressive potential of these cells. Incubation with IFN-gamma for 24 hours had no effect on the progenitor cells of fetal origin (Fig. 1E). However, the adult progenitor cells perform better after the IFN-gamma treatment, which is especially clear in the case of the adult CMPC ($p = 0.006$).

3.2. BM-MSC and CMPC alter the inflammatory environment of stimulated T cells

To examine the production and release of cytokines, conditioned medium (CM) was collected from individual BM-MSC, CMPC, and T cell cultures and from co-

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cultures of BM-MSC or CMPC with T cells. Cytokines indicative of both T_H1 and T_H2 were significantly reduced in the presence of BM-MSC and CMPC (Fig. 3A; Table 1). Interleukin-10 (IL-10), usually suggested to be anti-inflammatory and an inducer of T_{reg}, was produced in high levels by active and proliferating T cells, whereas its release was strongly diminished in the presence of BM-MSC or CMPC ($p < 0.001$ for both; Fig. 3B). The cytokine IL-1beta was produced by BM-MSC and in high levels by CMPC compared to stimulated T cells ($p < 0.01$). In the co-culture experiments, these levels were increased even further, to 13-fold for BM-MSC and 52-fold for CMPC ($p < 0.001$).

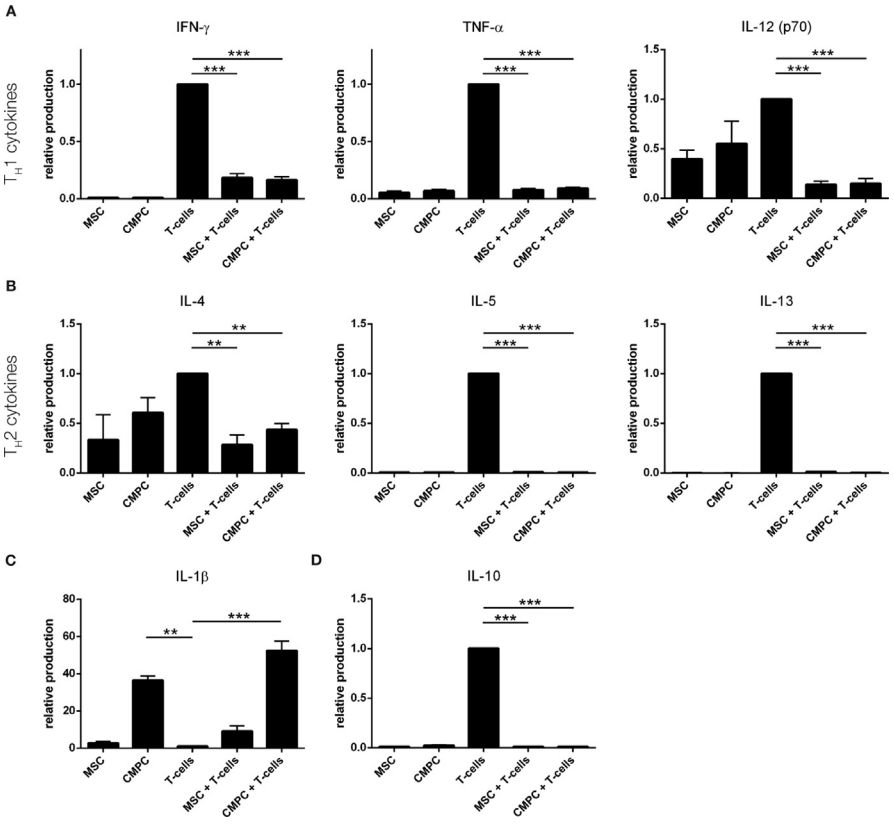

Fig. 3. Alteration of inflammatory environment. The cytokine production by BM-MSC and CMPC only, by T cells only, and upon co-culture of stem cells and T cells was measured by Luminex assays. A. Pro-inflammatory T_H1 cytokines cytokines IFN-gamma, TNF-alpha and IL-12 are produced by stimulated T cells but down-regulated upon co-culturing with both BM-MSC and CMPC. A significant reduction in these cytokines in the conditioned medium indicated an environment that does not support the development of T_H1 cells. B. The development of T_H2 cells is determined by the presence of IL-4, IL-5 and IL-13. These cytokines are all upregulated in response to T cell activation and are significantly suppressed in the presence of progenitor cells. C. The release of IL-1b, which is produced by BM-MSC and CMPC yet hardly by T cells, is further increased upon co-culture with T cells. D. Release of IL-10, a supposedly anti-inflammatory cytokine, is strongly suppressed when progenitor cells are present in the co-culture, yet highly produced by stimulated T cells. For values see also Table 1. ***$p < 0.001$ and **$p < 0.01$.

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Table 1. Alteration of inflammatory environment.

Cytokine release										
		IL-1β	**IL-2**	**IL-4**	**IL-5**	**IL-6**	**IL-10**	**IL-12-p70**	**IL-13**	**IFN-gamma**	**TNF-alpha**
T-cells	unstimulated	3.64 ± 2.51 p = 1.000	0.01 ± 0.01 p = 1.000	0.08 ± 0.02 p < 0.001	0.00 ± 0.00 p < 0.001	0.26 ± 0.14 p < 0.001	0.01 ± 0.01 p < 0.001	0.04 ± 0.03 p < 0.001	0.00 ± 0.00 p < 0.001	0.00 ± 0.00 p < 0.001	0.02 ± 0.01 p < 0.001
	stimulated	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
BM-MSC	unstimulated	0.43 ± 0.06 p = 1.000	0.24 ± 0.17 p = 1.000	0.33 ± 0.26 p = 0.002	0.01 ± 0.00 p < 0.001	1.25 ± 0.08 p = 0.495	0.01 ± 0.00 p < 0.001	0.37 ± 0.04 p = 0.001	0.00 ± 0.00 p < 0.001	0.01 ± 0.00 p < 0.001	0.04 ± 0.00 p < 0.001
	stimulated	2.74 ± 0.85 p = 1.000	27.24 ± 18.15 p = 0.070	0.33 ± 0.25 p = 0.002	0.01 ± 0.00 p < 0.001	1.25 ± 0.11 p = 0.476	0.01 ± 0.00 p < 0.001	0.40 ± 0.09 p = 0.002	0.00 ± 0.00 p < 0.001	0.01 ± 0.00 p < 0.001	0.05 ± 0.01 p < 0.001
CMPC	unstimulated	5.86 ± 2.34 p = 0.996	0.01 ± 0.00 p = 1.000	0.50 ± 0.12 p = 0.051	0.01 ± 0.00 p < 0.001	1.09 ± 0.03 p = 0.999	0.02 ± 0.01 p < 0.001	0.50 ± 0.19 p = 0.017	0.00 ± 0.00 p < 0.001	0.01 ± 0.00 p < 0.001	0.06 ± 0.01 p < 0.001
	stimulated	36.49 ± 2.32 p = 0.005	1.03 ± 0.22 p = 1.000	0.61 ± 0.15 p = 0.233	0.01 ± 0.00 p < 0.001	1.09 ± 0.04 p = 0.999	0.03 ± 0.00 p < 0.001	0.55 ± 0.23 p = 0.047	0.00 ± 0.00 p < 0.001	0.01 ± 0.00 p < 0.001	0.07 ± 0.01 p < 0.001
BM-MSC + T-cells	unstimulated	0.51 ± 0.12 p = 1.000	0.22 ± 0.07 p = 1.000	0.21 ± 0.08 p < 0.001	0.00 ± 0.00 p < 0.001	1.26 ± 0.07 p = 0.123	0.01 ± 0.00 p < 0.001	0.17 ± 0.13 p < 0.001	0.00 ± 0.00 p < 0.001	0.01 ± 0.00 p < 0.001	0.05 ± 0.02 p < 0.001
	stimulated	9.13 ± 2.93 p = 0.689	31.71 ± 10.07 p = 0.001	0.28 ± 0.10 p = 0.005	0.01 ± 0.00 p < 0.001	1.29 ± 0.07 p = 0.065	0.01 ± 0.00 p < 0.001	0.14 ± 0.03 p < 0.001	0.01 ± 0.00 p < 0.001	0.18 ± 0.04 p < 0.001	0.08 ± 0.01 p < 0.001
CMPC + T-cells	unstimulated	8.10 ± 1.84 p = 0.777	0.01 ± 0.00 p = 1.000	0.25 ± 0.03 p < 0.001	0.00 ± 0.00 p < 0.001	1.09 ± 0.02 p = 0.983	0.06 ± 0.02 p < 0.001	0.12 ± 0.04 p < 0.001	0.00 ± 0.00 p < 0.001	0.01 ± 0.00 p < 0.001	0.06 ± 0.01 p < 0.001
	stimulated	52.41 ± 5.21 p < 0.001	1.17 ± 0.07 p = 1.000	0.44 ± 0.06 p = 0.005	0.01 ± 0.00 p < 0.001	1.07 ± 0.02 p = 0.997	0.01 ± 0.00 p < 0.001	0.15 ± 0.049 p < 0.001	0.00 ± 0.00 p < 0.001	0.16 ± 0.03 p < 0.001	0.09 ± 0.01 p < 0.001

Table showing the fold-increase or decrease in cytokine production compared to the stimulated T-cells. P-values are shown where applicable.
IL = Interleukin, IFN-gamma = interferon-gamma, TNF-alpha = tumor necrosis factor-alpha, MSC = mesenchymal stem cells, CMPC = cardiomyocyte progenitor cells.

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3.3. Paracrine factors are responsible for T cell suppression

To investigate the dependence on direct cell-cell contact, we used an insert during culture to separate the different cell types from each other. Inhibitions of cell-contact did not reduce the immunomodulative effects (BM-MSC: 58 ± 10%, CMPC: 62 ± 9%, $p < 0.05$ for both; Fig. 4A). No statistically significant differences were observed between the groups with or without transwell filter.

Subsequently, we collected conditioned medium (CM) after six days of single- or co-culture, to investigate if a soluble, stable compound was present in cellular secretions. The obtained CM was added 1:1 with fresh medium to newly isolated and stimulated T cells. A significant suppression of T cell proliferation occurred in the presence of CM obtained from BM-MSC (40 ± 10%, $p < 0.01$), while the CM from the co-culture of BM-MSC with T cells performed even better (51 ± 8%, $p < 0.01$; Fig. 4B). This difference was, however, not significant. Even more pronounced

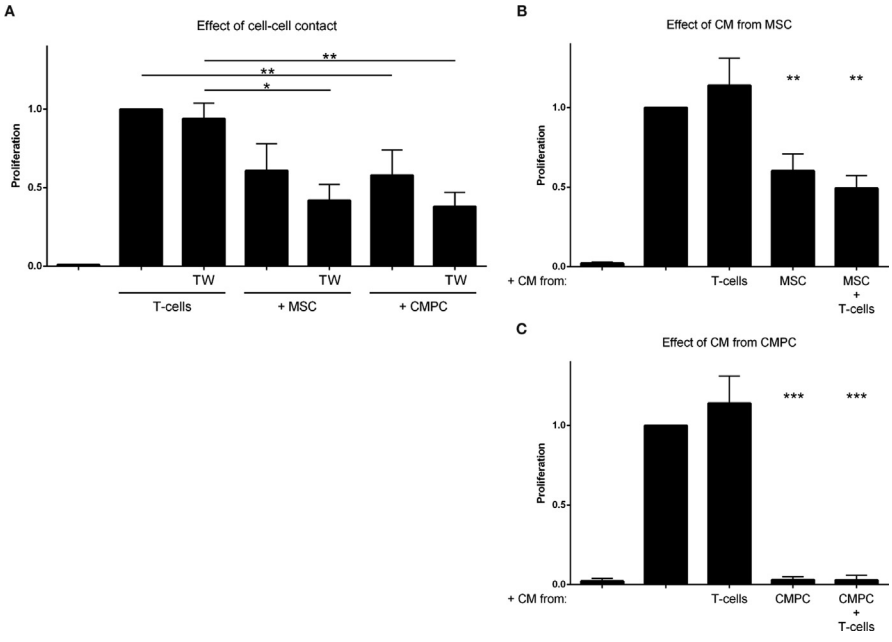

Fig. 4. Paracrine effect. A. Transwell experiment (TW) were performed where BM-MSC or CMPC are located in the bottom part and activated T cells on top of the 0.4 μm TW-filter. In control groups the cells were allowed cell-cell contact. A. Stimulated T cell co-cultures with BM-MSC and CMPC (TW −) and without (TW +) direct cell-cell contact. Suppression of proliferation occurs in cell contact groups (BM-MSC: 39 ± 17%, n.s., CMPC: 42 ± 16%, $p < 0.05$). Reduction of proliferation still occurs in the absence of direct cell-cell interactions (BM-MSC: 58 ± 10%, CMPC: 62 ± 9%, $p < 0.05$ for both) ($n = 5$). A. Stimulated T cells grown in the presence of CM from BM-MSC or the BM-MSC-T cell co-culture have a significantly reduced proliferation (40 ± 10% ($n = 3$) and 51 ± 10% ($n = 7$), respectively), whereas CM from stimulated T cell has no effect (14 ± 17% increase). B. Stimulated T cells proliferate significantly less after addition of CM from CMPC (97 ± 0.2%, $p < 0.0001$) or the CMPC-T cell co-culture (97 ± 0.3%). ***$p < 0.001$, **$p < 0.01$ and *$p < 0.05$.

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effects were observed with the CMPC, where the CM from both the CMPC and the co-culture completely suppressed T cell proliferation ($p < 0.0001$) (Fig. 4C). No significant effect, neither suppressive nor stimulatory, was seen after addition of CM derived from stimulated T cells only (1.14 ± 17%, Fig. 4B and C).

3.4. Stem cell derived extracellular vesicles can inhibit T cell proliferation

To investigate whether extracellular vesicles (EVs) secreted by stem cells can influence the proliferation of T cells, we isolated EVs from conditioned medium of BM-MSC and CMPC. To demonstrate uptake of EVs by stimulated T cells, we added purified PKH-26 (red) labeled EVs to CFSE-labeled (green) T cells. After overnight incubation, we observed by fluorescence microscopy a clear co-localization of the exosome-, T cell- and nuclear labels (Fig. 5A). We then quantified the uptake of these EVs by T cells over time (Fig. 5B) and noticed most EV uptake occurred in the first hour after addition. Around 35% of the T cells is positive at this time-point, which rises to approximately 60% after 24 hours. Control stainings were performed that suggest that free PKH-26 dye, obtained after ultracentrifugation, did not lead to signal uptake.

Next, we examined the effect of EVs on T cell proliferation in a dose-response experiment using 0.0025μg−10μg of EVs (Fig. 5C). A clear dose-dependent effect on T cell proliferation was visible, as shown in Fig. 5C. We subsequently added 1.5μg of BM-MSC- or CMPC-derived EVs to stimulated T cells. The addition of BM-MSC- or CMPC-derived EVs resulted in a strong decrease of proliferation (Fig. 5D; 73 ± 12%, $p < 0.01$ and 77 ± 10%, $p < 0.01$, respectively).

3.5. Pathway inhibitors

Several studies have reported a number of pathways to be involved in the immunosuppressive responses, amongst which indoleamine 2,3-dioxygenase (IDO) and prostaglandin E2 (PGE2) [14,41,44,45]. However, we studied the proteomics of our EVs and did not find any components of the IDO or PGE2 pathways to be present (data not shown). Even so, we performed the co-culture assay in the presence of several inhibitors: 1-methyl-L-tryptophan (against IDO), indomethacine (against PGE2) and Alk5 (against TGF-beta). Upon co-culture we did not see any change in the T cell suppression (Fig. 6). In addition to the inhibitor doses used by other research groups [46, 47, 48], we have performed a dose titration, as well as preconditioning and repetitive administration of the inhibitors to block the immunosuppression. However, we still did not find any effect of these inhibitors.

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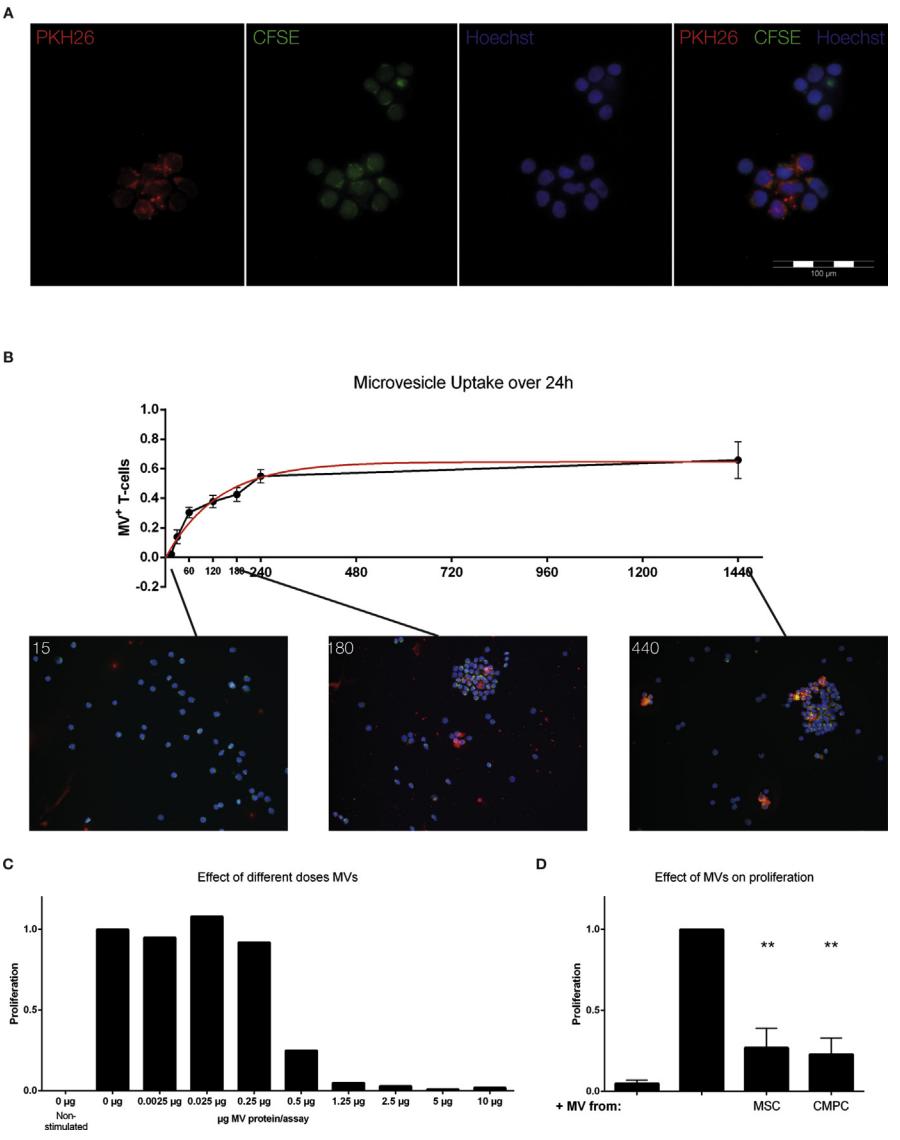

Fig. 5. Effect of stem cell derived EVs on T cell proliferation. EVs were isolated from the conditioned medium of CMPC and BM-MSC to investigate their immunomodulative potential. A. Fluorescence microscope visualization of the EV uptake by T cells. Red: EVs (PKH-26). Green: T cells (CFSE). Blue: nucleus (Hoechst). B Titration curve of CMPC derived EV protein concentration and the effect on T cell proliferation after stimulation. A cut-off point is reached around 1 µg of EV protein. C BM-MSC- and CMPC-derived EVs significantly reduce T cell proliferation (BM-MSC-EV: 73 ± 12%, CMPC-EV: 77 ± 10%). **$p < 0.01$ compared to stimulated T cell.

3.6. RNAseq shows differentially regulated genes

In order to get an unbiased view on the changes induced in the T cells, we performed an RNA sequence on several individual T cell donors that were exposed to different progenitor cell donors.

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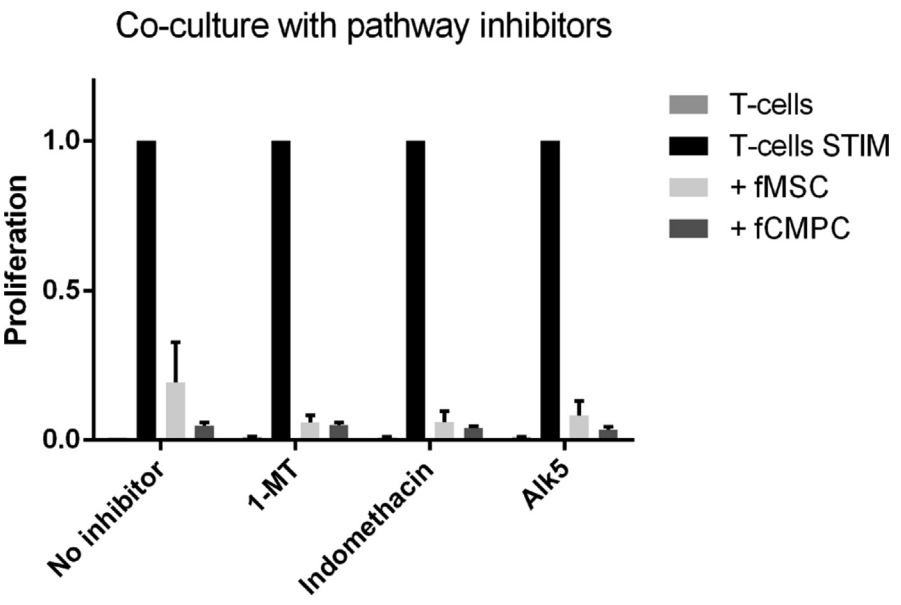

Fig. 6. Effect of inhibitors on immunosuppression. Co-culture was performed as described, in the presence or absence of progenitor cells and inhibitors for the immunosuppressive pathways: 1-MT (1 mM; blocks IDO), Indomethacin (10 μM; block PGE2) and Alk5 (10 μM: competes with TGF-beta signaling). None of these inhibitors showed an effect compared to the control group. This is one representative set of many experiments, which all showed no effect on T cell proliferation.

Unsupervised principal component analysis in the sixteen T cell samples, color-coded by group (Fig. 7A) based on 5000 most variable transcripts (ref DESeq2), illustrated a close proximity of samples per group. Additionally, principal component analysis showed that 66% of the variation in the dataset can be explained by either the presence of the progenitor cells (PC1; 40%) and the T cell activation (PC2; 26%).

Upon activation of T cells, almost 1500 genes were significantly upregulated (>2-fold, padj < 0.05). Of these genes, 416 were suppressed (>2-fold) in the presence of BM-MSC, compared to only 100 genes in the presence of CMPC (Fig. 7B). 86 T cell genes were upregulated upon activation and suppressed in the presence of either BM-MSC or CMPC, as shown in the Venn diagram (see also Table 2). Subsequently, we entered these genes in IPA, which showed pathways concerning inflammation and immune cell proliferation. However, we noticed all these pathways depended largely on a small number of the 86 genes. Therefore, we used the NBCI Gene database (http://www.ncbi.nlm.nih.gov/gene/) and the GeneCards database (http://www.genecards.org/) to investigate the function of these genes in T cells. As depicted in the pie-chart in Fig. 7C, 20% of these genes (17 genes) has a known role in cell proliferation. Another 14% (12 genes) is related to the production and release of cytokines and/or their receptors. Lastly, only 3 genes (~3%) are associated with inflammation. This leaves 54 genes (63%) that have no clear role in proliferation/inflammation or no known function.

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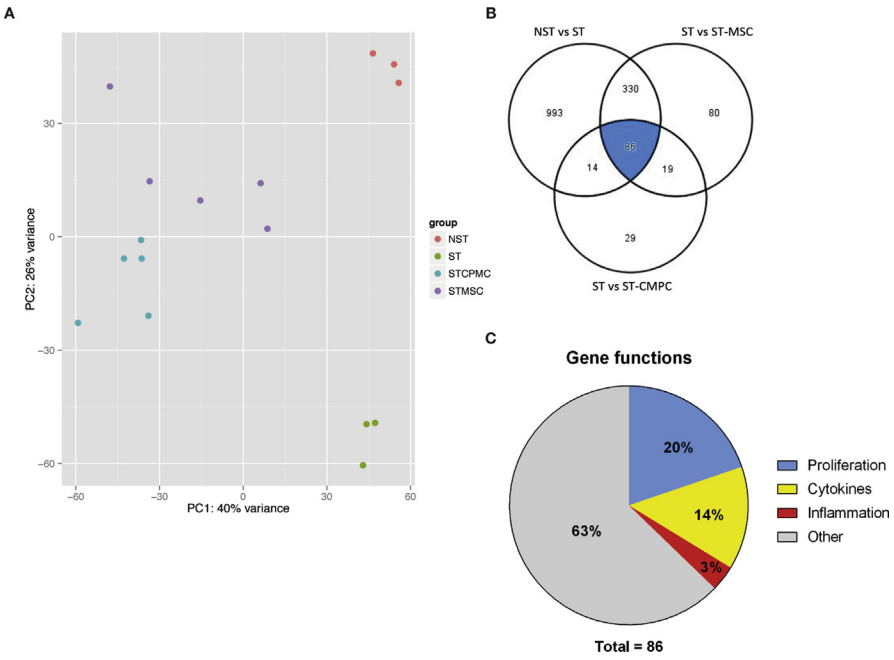

Fig. 7. RNAseq of T cell modulation. A. Principal component analysis shows a division of samples in four groups, matching our experimental conditions. Most variation between the samples is explained by the presence of stem cells in the culture (PC1: 40%) and the activation of T cells (PC2: 26%) (NST – non stimulated, ST stimulated etc) B. Venn-diagram showing the overlap between genes that are >2-fold up-regulated upon activation (left-upper circle), and >2-fold downregulated in the vicinity of BM-MSC (right-upper circle) or CMPC (lower circle). C. Pie-chart showing the functions of the 86 genes found.

4. Discussion

In this study, we have demonstrated that both BM-MSC and CMPC inhibit T cell proliferation *in vitro*, with CMPC having a significantly stronger suppressive effect than BM-MSC. There was no difference in low and high passage progenitor cells on suppressing T cell proliferation (data not shown) but CMPC showed differences based on donor age. In addition to suppressing proliferation, both BM-MSC and CMPC also suppressed the release of pro-inflammatory cytokines from T cells. Although a shift towards a specific T cell phenotype is occasionally reported [35, 49], we found cytokines of both T_H1, T_H2 and T_{reg} subtypes to be suppressed, suggesting that the T cells remain in a naïve, inactivated state.

It was interesting to notice both BM-MSC and CMPC produce high levels of IL-1beta, when compared to the stimulated T-cells. Although it is known cardiomyocytes can produce IL-1 under ischemic stress, this has not yet been observed for these progenitors [50]. It could be hypothesized that inflammatory stress might trigger CMPC to produce IL-1, thereby potentially stimulating inflammation. However, a recent study showed IL-1 actually primes MSC to maintain an anti-inflammatory phenotype [51]. Therefore, one could also argue that progenitor cells produce this

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Table 2. 86 altered genes.

	Symbol	Entrez Gene Name	Location	Type(s)
1	ASB2	ankyrin repeat and SOCS box containing 2	Nucleus	transcription regulator
2	ASB9	ankyrin repeat and SOCS box containing 9	Nucleus	transcription regulator
3	DDX4	DEAD (Asp-Glu-Ala-Asp) box polypeptide 4	Nucleus	enzyme
4	DEPDC1	DEP domain containing 1	Nucleus	transcription regulator
5	DHRS2	dehydrogenase/reductase (SDR family) member 2	Nucleus	enzyme
6	EBNA1BP2	EBNA1 binding protein 2	Nucleus	other
7	GINS2	GINS complex subunit 2 (Psf2 homolog)	Nucleus	other
8	HIST1H1A	histone cluster 1, H1a	Nucleus	other
9	HIST1H2AI	histone cluster 1, H2ai	Nucleus	other
10	HIST1H2BC	histone cluster 1, H2bc	Nucleus	other
11	HIST1H2BL	histone cluster 1, H2bl	Nucleus	other
12	HIST1H3J	histone cluster 1, H3j	Nucleus	other
13	HIST2H2AB	histone cluster 2, H2ab	Nucleus	other
14	HIST2H2BF	histone cluster 2, H2bf	Nucleus	other
15	HIST3H2BB	histone cluster 3, H2bb	Nucleus	other
16	MCM2	minichromosome maintenance complex component 2	Nucleus	enzyme
17	POLR3G	polymerase (RNA) III (DNA directed) polypeptide G (32kD)	Nucleus	enzyme
18	RANBP1	RAN binding protein 1	Nucleus	other
19	S100A2	S100 calcium binding protein A2	Nucleus	other
20	TERT	telomerase reverse transcriptase	Nucleus	enzyme
21	APOBEC3B	apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3B	Cytoplasm	enzyme
22	BSPRY	B-box and SPRY domain containing	Cytoplasm	other
23	CAMK1	calcium/calmodulin-dependent protein kinase I	Cytoplasm	kinase
24	CCNB1	cyclin B1	Cytoplasm	kinase
25	DAPP1	dual adaptor of phosphotyrosine and 3-phosphoinositides	Cytoplasm	other
26	GAD1	glutamate decarboxylase 1 (brain, 67kDa)	Cytoplasm	enzyme
27	GALNT18	polypeptide N-acetylgalactosaminyltransferase 18	Cytoplasm	enzyme
28	GLDC	glycine dehydrogenase (decarboxylating)	Cytoplasm	enzyme

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Table 2. (*Continued*)

	Symbol	Entrez Gene Name	Location	Type(s)
29	KLK1	kallikrein 1	Cytoplasm	peptidase
30	MB	myoglobin	Cytoplasm	transporter
31	NCF2	neutrophil cytosolic factor 2	Cytoplasm	enzyme
32	NME1	NME/NM23 nucleoside diphosphate kinase 1	Cytoplasm	kinase
33	ODF1	outer dense fiber of sperm tails 1	Cytoplasm	other
34	PAICS	phosphoribosylaminoimidazole carboxylase, phosphoribosylaminoimidazole succinocarboxamide synthetase	Cytoplasm	enzyme
35	PLCG2	phospholipase C, gamma 2 (phosphatidylinositol-specific)	Cytoplasm	enzyme
36	PTPN3	protein tyrosine phosphatase, non-receptor type 3	Cytoplasm	phosphatase
37	SERPINB10	serpin peptidase inhibitor, clade B (ovalbumin), member 10	Cytoplasm	other
38	STON2	stonin 2	Cytoplasm	other
39	TPRG1	tumor protein p63 regulated 1	Cytoplasm	other
40	ART3	ADP-ribosyltransferase 3	Plasma Membrane	enzyme
41	CCR2	chemokine (C-C motif) receptor 2	Plasma Membrane	G-protein coupled receptor
42	CHRNA6	cholinergic receptor, nicotinic, alpha 6 (neuronal)	Plasma Membrane	transmembrane receptor
43	CLECL1	C-type lectin-like 1	Plasma Membrane	other
44	CPNE5	copine V	Plasma Membrane	other
45	ENPP2	ectonucleotide pyrophosphatase/ phosphodiesterase 2	Plasma Membrane	enzyme
46	GAP43	growth associated protein 43	Plasma Membrane	other
47	GJB2	gap junction protein, beta 2, 26kDa	Plasma Membrane	transporter
48	GNA14	guanine nucleotide binding protein (G protein), alpha 14	Plasma Membrane	enzyme
49	HCAR1	hydroxycarboxylic acid receptor 1	Plasma Membrane	G-protein coupled receptor
50	IGHM	immunoglobulin heavy constant mu	Plasma Membrane	transmembrane receptor
51	IL17RB	interleukin 17 receptor B	Plasma Membrane	transmembrane receptor
52	IL23R	interleukin 23 receptor	Plasma Membrane	transmembrane receptor
53	MYO3B	myosin IIIB	Plasma Membrane	kinase
54	NINJ2	ninjurin 2	Plasma Membrane	other
55	TNFRSF8	tumor necrosis factor receptor superfamily, member 8	Plasma Membrane	transmembrane receptor

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Table 2. (*Continued*)

	Symbol	Entrez Gene Name	Location	Type(s)
56	CGREF1	cell growth regulator with EF-hand domain 1	Extracellular Space	other
57	EBI3	Epstein-Barr virus induced 3	Extracellular Space	cytokine
58	FBLN5	fibulin 5	Extracellular Space	other
59	IFNG	interferon, gamma	Extracellular Space	cytokine
60	IL17A	interleukin 17A	Extracellular Space	cytokine
61	IL17F	interleukin 17F	Extracellular Space	cytokine
62	IL5	interleukin 5	Extracellular Space	cytokine
63	IL9	interleukin 9	Extracellular Space	cytokine
64	LTA	lymphotoxin alpha	Extracellular Space	cytokine
65	NAPSA	napsin A aspartic peptidase	Extracellular Space	peptidase
66	PRG4	proteoglycan 4	Extracellular Space	other
67	TNFSF15	tumor necrosis factor (ligand) superfamily, member 15	Extracellular Space	cytokine
68	C4orf26	chromosome 4 open reading frame 26	Other	other
69	CDC20P1	cell division cycle 20 pseudogene 1	Other	other
70	CKS2	CDC28 protein kinase regulatory subunit 2	Other	kinase
71	COX17P1	COX17 cytochrome c oxidase copper chaperone pseudogene 1	Other	other
72	HMSD	histocompatibility (minor) serpin domain containing	Other	other
73	HPDL	4-hydroxyphenylpyruvate dioxygenase-like	Other	other
74	HSPE1P2	heat shock 10kDa protein 1 pseudogene 2	Other	other
75	LINC00158	long intergenic non-protein coding RNA 158	Other	other
76	LINC00877	long intergenic non-protein coding RNA 877	Other	other
77	LINC00892	long intergenic non-protein coding RNA 892	Other	other
78	LINC01132	long intergenic non-protein coding RNA 1132	Other	other
79	LINC01281	long intergenic non-protein coding RNA 1281	Other	other
80	NAPSB	napsin B aspartic peptidase, pseudogene	Other	other
81	PAICSP4	phosphoribosylaminoimidazole carboxylase, phosphoribosylaminoimidazole succinocarboxamide synthetase pseudogene 4	Other	other
82	PHBP3	prohibitin pseudogene 3	Other	other
83	PIK3CD-AS2	PIK3CD antisense RNA 2	Other	other
84	RCAN2	regulator of calcineurin 2	Other	other
85	RNU5A-8P	RNA, U5A small nuclear 8, pseudogene	Other	other
86	SLC16A9	solute carrier family 16, member 9	Other	other

This table contains the 86 genes significantly upregulated by activation of T cells, and significantly downregulated in the presence of BM-MSC or CMPC. It shows the symbol, name, location and the type of the end-product.

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cytokine to control their own function in inflammatory conditions. More studies on this will be needed to further elicit the processes at play here.

Suppression of T cell proliferation by BM-MSC has been shown in previous studies [24, 25, 26, 46, 52], albeit with strong variation in suppression between different donors [52]. With our discovery that CMPC are strong modulators of the immune system and the reports of immunomodulative effects of neural stem cells [52, 53], we wondered whether immunomodulation is a more general stem cell trait. For this reason, we included ECFC, a circulatory endothelial colony forming cell, which proved unable to alter the number of proliferating cells.

Both BM-MSC and CMPC mediated immunomodulation by production of paracrine factors, which was readily demonstrated in our transwell experiments. This finding is in agreement with DiNicola *et al.* [24] who also found that cell-cell contact was dispensable for immunosuppression by BM-MSC, while others [41, 46, 54] found reduced suppression in absence of cell-contact. A possible explanation for this is that cell-cell contact is not necessary for the suppression itself, but for induction of the T-regulatory phenotype [41]. Another explanation for these observed differences could be the different origins, isolation methods and (co-)culture methods in the different studies, which makes it hard to compare an already heterogenous group of progenitor cells [52].

The immunomodulative paracrine factors were already present during normal expansion cultures without any immune cells being present (Figs. 4 and 5). This is in contrast with studies that claim the progenitor cells need to be 'licensed' by immune cells to release these suppressive factor [41, 42, 43]. Again, it is quite likely that the different origins of progenitor cells in different studies could be responsible there. Our study shows that adult progenitor cells appear to diminish in immunosuppressive capacity, but can be re-activated by exposing them to IFN-gamma, while on the fetal progenitor cells this preconditioning has no effect.

Immunosuppression by EVs derived from (modified) dendritic cells [55, 56, 57] or cancer stem cells [58] has been reported in the past. Meanwhile, more recent studies have shown effects of BM-MSC-derived EVs on inflammation in different organ tissues and proliferation of lymphocytes [21, 29, 30, 59, 60, 61, 62]. In many of these studies either an animal model or the whole peripheral blood mononuclear cell (PBMC) or splenocyte fraction was used, leaving it ambiguous whether observed effects were caused by direct interference with the T cells or indirectly via another cell type, such as macrophages [29, 30, 61, 63]. Only few other studies directly look at a suppressive effect on T-cells [21, 64]. We examined the immunomodulating capacity of not only BM-MSC-derived exosomes, but also the CMPC-derived EVs on pure CD3+ T cells and observed a strong inhibition of proliferation *in vitro* when either EVs was added to stimulated T cells. Our CMPC-EV titration experiment indicated this effect is dose-dependent, as was also observed by others for MSC-EVs

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[21]. However, the amount of suppression differs between the different studies, likely due to different culture and isolation methods, as well as subtle differences in amounts of EVs added. We do believe that, although BM-MSC- and CMPC-derived EVs are important mediators of immunomodulation, they do not cover the complete suppressive effect, and will most likely function optimally in combination with several growth factors or cytokines produced by the progenitor cells.

Several potential mediators have been investigated for their involvement in the immunomodulatory effects, including interleukin-10 (IL-10), inducible nitric oxide synthase (iNOS), transforming growth factor-beta (TGF-β), prostaglandin E2, and indoleamine 2,3-dioxygenase (IDO) [14, 25, 41, 44, 46, 65]. Of these, the last two have been most investigated in different settings. Several studies have attempted to block these pathways, often resulting in a variable decrease of the immune suppressive effect of BM-MSC. However, these experiments had variable outcomes and until now the exact mechanisms of immune suppression remain controversial [14, 41, 46, 65, 66]. In our hands, addition of inhibitors for these pathways did not show any effect on the immunosuppressive effects of the progenitor cells. We did not include an inhibitor against iNOS in these experiments, as our CM experiments already demonstrated the mediator is a stable compound, which nitric oxide (NO) is not.

An explanation for our observed ineffectiveness of pathway inhibition is suggested by our RNA sequencing. We found 86 genes which are upregulated during T cell activation and are suppressed in the presence of progenitor cells. Less than half of these genes is directly linked to proliferation or inflammation; the majority has either completely different or unknown functions. We believe these genes to play an important role in the modulation of T cells and warrant further investigation.

We recognize some limitations of this study. The first is inherent in the study of the immune system *in vitro*. We used a non-physiological method of T cell activation, as the complex activation seen after MI is impossible to simulate in vitro [14, 67]. This leads directly to the second limitation in our study, which applies to all *in vitro* immune research: the immune system is a complex and interactive system in which all components strongly influence each other and excluding a specific cell type could unbalance this system and possibly influence the interactions with BM-MSC or CMPC. Thirdly, the exact murine counterpart of the human cardiac-derived CMPC has not yet been identified. Therefore, *in vivo* research using human CMPC is exclusively performed in immunodeficient mice to reduce immediate stem cell rejection. Unfortunately, this also prevents the investigation of T-cell responses upon stem cell injections after MI, as these animals have no adaptive immune system. A humanized mouse model would be necessary to confirm the in vivo potential of these cells is as strong as observed here *in vitro*.

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We demonstrated that both mesenchymal stem cells (BM-MSC) and cardiomyocyte progenitor cells (CMPC) strongly modulate the immune system by attenuating T cell proliferation *in vitro* and reducing release of pro-inflammatory cytokines. This suppression is not dependent on 'licencing' nor on cell-cell contact. It is mediated via paracrine factors, which are already produced during regular culture. EVs isolated from the conditioned medium were shown to be dose-dependently capable of suppressing T cell proliferation and should be further studied as a possible new treatment for post-MI inflammation, to reduce damage to the heart in both short and long term. Lastly, despite earlier publication on pathways involved, we found a pallet of unstudied genes expected to play a major role in the activation and suppression of T cells which need further investigation.

Declarations

Author contribution statement

Frederieke van den Akker: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Krijn Vrijsen: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data.

Janine Deddens: Performed the experiments; Analyzed and interpreted the data.

Jan-Willem Buikema, Linda Van Laake: Analyzed and interpreted the data; Wrote the paper.

Michal Mokry, Pieter Doevendans: Analyzed and interpreted the data.

Joost Sluijter: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

Supplementary content related to this article has been published online at https://doi.org/10.1016/j.heliyon.2018.e00642.

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