

Apoptotic mesenchymal stromal cells induce prostaglandin E2 in monocytes: implications for the monitoring of mesenchymal stromal cell activity

Tik Shing Cheung,¹ Antonio Galleu,¹ Malte von Bonin,² Martin Bornhäuser² and Francesco Dazzi¹

¹School of Cancer and Pharmacological Sciences and KHP Cancer Research UK Centre, King's College London, London, United Kingdom; ²University Hospital Carl Gustav Carus, Dresden, Germany

*Correspondence: FRANCESCO DAZZI - francesco.dazzi@kcl.ac.uk
doi:10.3324/haematol.2018.214767*

Supplementary Materials to:

**Apoptotic mesenchymal stromal cells induce prostaglandin E₂ in monocytes:
implications for the monitoring of mesenchymal stromal cell activity**

Tik Shing Cheung¹, Antonio Galleu¹, Malte von Bonin², Martin Bornhäuser²,
Francesco Dazzi¹

¹School of Cancer and Pharmacological Sciences and KHP Cancer Research UK
Centre, King's College London, London, United Kingdom. ²University Hospital
Carl Gustav Carus, Dresden, Germany.

Corresponding author:

Francesco Dazzi

School of Cancer and Pharmacological Sciences and KHP Cancer Research UK
Centre

The Rayne Institute

King's College London

123, Coldharbour Lane

London SE5 9NU

francesco.dazzi@kcl.ac.uk

Supplementary Methods

MSC isolation and culture

Human bone marrow-derived MSCs were isolated from the bone marrow aspirate taken from the iliac crest of healthy donor, which was obtained from the Imperial College Healthcare Tissue Bank (ICHTB, HTA license 12275). ICHTB is supported by the National Institute for Health Research (NIHR) Biomedical Research Centre based at Imperial College Healthcare NHS Trust and Imperial College London. ICHTB is approved by the UK National Research Ethics Service to release human material for research (12/WA/0196), and the samples for this project were issued from the sub-collection. Informed consent was obtained in accordance with ICHTB requirements and procedures were carried out in accordance with relevant guidelines and regulations. Mononuclear cells were first plated ($10 - 25 \times 10^6$ per 636 cm^2) in MEM- α (Thermo Fisher Scientific) containing 5% (v/v) human platelet lysate (Cook Regentec) and 1 U/mL heparin (Wockhardt) at 37°C , 5% CO_2 . Non-adherent cells were removed after washing of PBS (Thermo Fisher Scientific) and replenishing the culture medium. When adherent MSCs reached approximately 70% to 90% confluence of culture flask, they were detached using Trypsin-EDTA (0.05%) with phenol red (Thermo Fisher Scientific) and expanded. MSCs at passage 3 to 7 (all from the same MSC donor) were used for experiments.

Generation of ApoMSCs

5×10^5 MSCs were treated with $10 \mu\text{g/mL}$ anti-Fas stimulating monoclonal antibody (CH11, Merck) in complete RPMI-1640 for 24 hours at 37°C , 5% CO_2 . Caspase activation was confirmed using pan-caspase inhibitor Z-VAD-FMK ($50 \mu\text{M}$, R&D Systems) under PE-Annexin V and 7-AAD (BD) staining using flow cytometry and caspase-3 (active) immunoblotting.

Protein extraction and Immunoblotting

Cell lysates from MSCs and ApoMSCs were homogenized using $100 \mu\text{L}$ radioimmunoprecipitation assay buffer with phenylmethanesulfonyl fluoride and protease inhibitors (all Thermo Fisher Scientific). After several vortexing and incubation on ice for 30 minutes, the protein extracts were obtained after centrifuging at $10,000 \text{ xg}$ for 15 mins at 4°C . The amount of protein was determined by bicinchoninic acid protein assay kit (Thermo Fisher Scientific).

Protein standards were prepared using bovine serum albumin (BSA) (Sigma–Aldrich) and deionized water. Amount of protein in samples was measured by NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific). Deionized water was used as blank. 20 µg protein per sample was prepared in Laemmli buffer (Thermo Fisher Scientific) and boiled at 100°C for 5 mins. Then, samples with equal protein amount were electrophoresed on 5–10% SDS–polyacrylamide gels and then transferred onto activated nitrocellulose membranes. After electroblotting, the membrane was washed by Tris–buffered saline–Tween20 (TBS–T) and 5 % (w/v) non–fat milk in TBS–T was used to block the membrane for 1 hour at room temperature. Discarding the blocking buffer, primary anti–caspase–3 (dilution 1:500, Cell Signaling Technology) in 3% BSA (w/v) TBS–T was added for overnight probing at 4°C. Anti–α–tubulin antibody (dilution 1:500, Santa Cruz Biotechnology) was used as protein loading control. Secondary antibody conjugated with horseradish peroxidase in 5% non–fat milk TBS–T (dilution 1:1000) was added for 1–hour incubation at room temperature followed by TBS–T washing. Protein expression was detected using Pierce™ ECL Western Blotting Substrate (Thermo Fisher Scientific). Membrane was imaged using Azure c300 Chemiluminescent Western Blot Imaging System.

Isolation of human PBMCs, monocytes and CD3 T cells

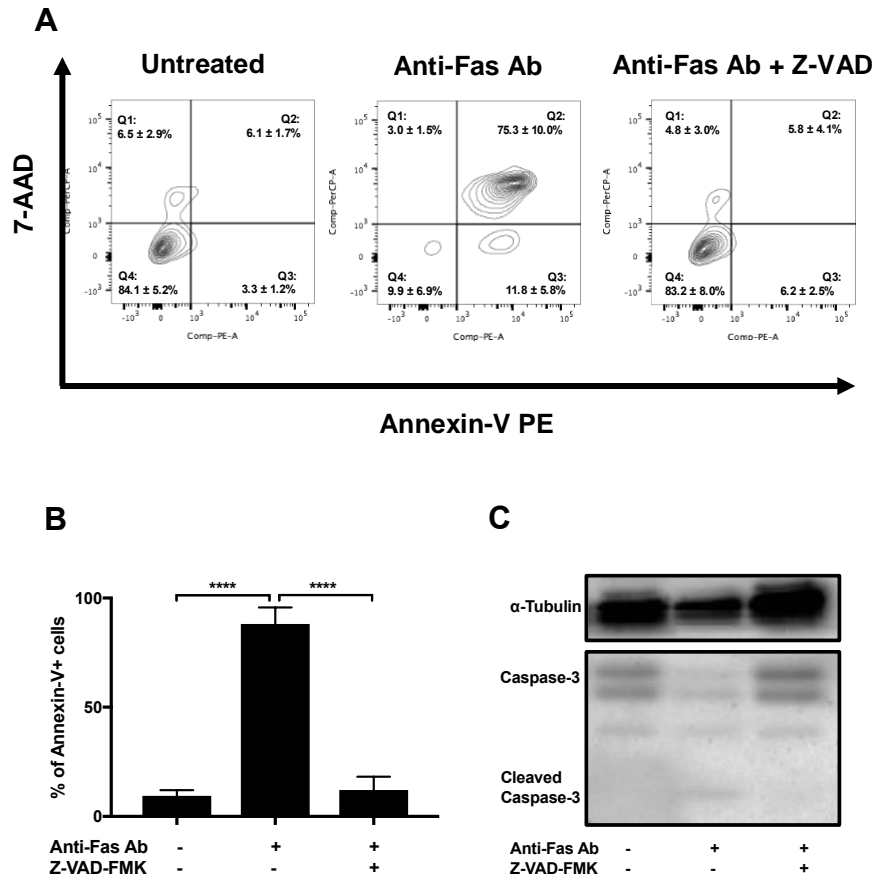
Human blood samples are purchased from the National Health Service (NHS) Blood and Transplant (UK) with the non–clinical account number (P243) for research purposes. For this project being conducted in England, no ethical approval is needed from the NHS Research Ethics Committee. Human peripheral blood mononuclear cells (PBMCs) were isolated from healthy volunteers' blood using Ficoll–Paque™ (Sigma–Aldrich). The storage of isolated human PBMCs is under the Human Tissue Authority (UK) license no.11023. To obtain monocytes and CD3 T cells, PBMCs were subjected to Pan Monocyte Isolation Kit II and CD3 MicroBeads (Miltenyi), respectively. After purification, CD14⁺ (61D3) cells were 91.9 ± 0.7 % (n=4), whilst CD3⁺ (UCHT1) cells were 96.7 ± 0.6% (n=3) compared to the isotype controls.

Statistical analysis

Statistical analysis was performed using PRISM version 6.0 (GraphPad, USA). Experimental data were expressed as mean ± SD from at least three

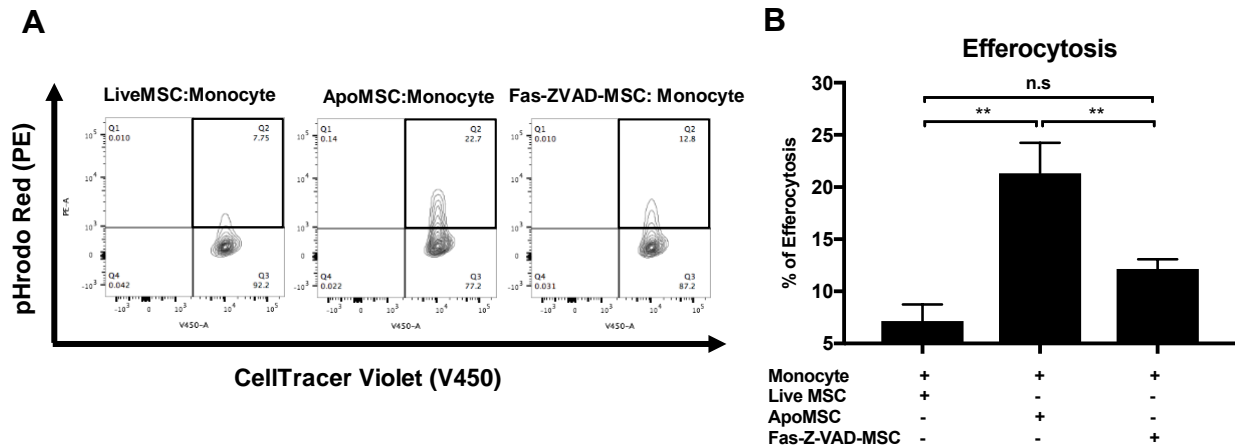
determinations. One-way ANOVA and post-hoc Tukey test were used to compare the mean differences when there are more than two samples. Unpaired t test was used to compare the mean differences between two samples. p values less than 0.05 were considered statistically significant.

Supplementary Figures



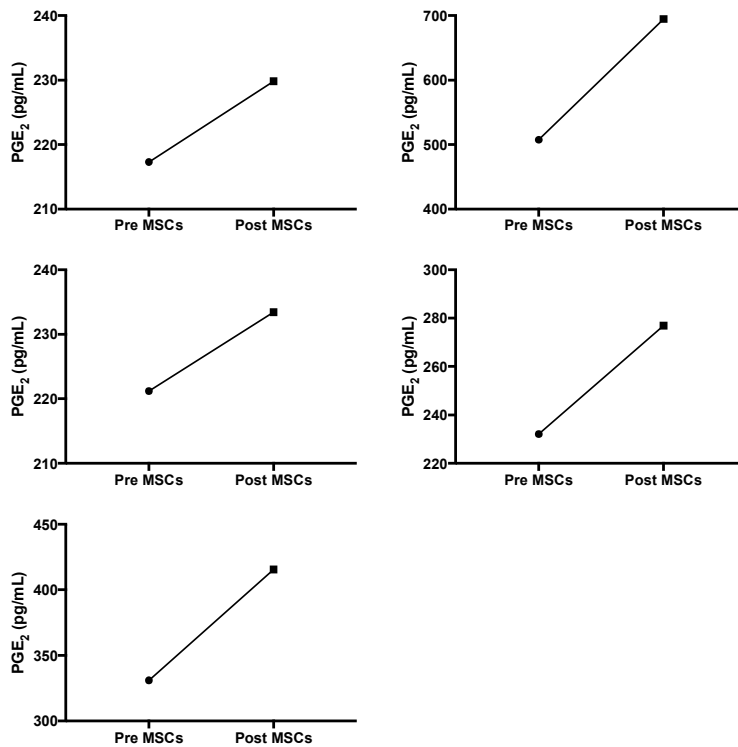
Supplementary Figure 1. Human bone marrow MSCs undergo Fas-stimulated apoptosis via caspase activation. (A) 5×10^5 MSCs were treated with $10 \mu\text{g}/\text{mL}$ anti-Fas stimulating monoclonal antibody (CH11) for 24 hours to induce apoptosis. Apoptosis was assessed using the PE-annexin-V and 7-AAD by flow cytometry. Gating strategy and representative plots annotated with experiment data (mean \pm SD in each quadrant) are shown, $n=5$. Pan-caspase inhibitor Z-VAD-FMK ($50\mu\text{M}$) was added during the incubation to examine the dependence of caspase activation following the Fas-stimulation (B). The percentage of apoptotic cells was calculated as Annexin-V⁺ cells by adding the percentage of cell population in the Q2 upper-right quadrant (Annexin-v⁺ and 7-AAD⁺) and Q2 lower-right quadrant (Annexin-v⁺ and 7-AAD⁻). Experimental data were expressed as mean \pm SD, $n=5$. One-way ANOVA and post-hoc Tukey test were used to compare the mean differences among the samples (**** p -values <

0.0001). (C) The protein lysates were extracted and performed immunoblotting with anti-caspase-3 (dilution 1:500) and anti- α -tubulin antibody (dilution 1:500). Dilution of secondary antibody conjugated with horseradish peroxidase was 1:1000. The image is the representative from 3 experiments.

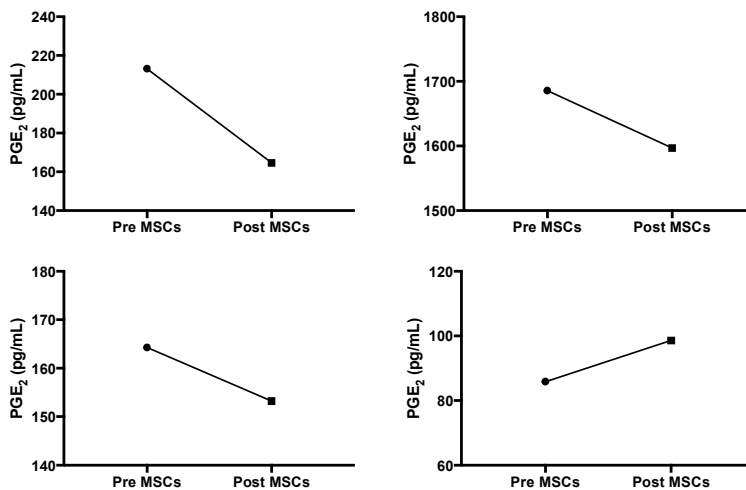


Supplementary Figure 2. Efferocytosis is selective for ApoMSCs. (A) LPS-activated monocytes were co-cultured with non-apoptotic MSCs (Live MSCs, untreated; Fas-ZVAD-MSC, MSCs treated with anti-Fas and caspase inhibitor Z-VAD-FMK) or ApoMSCs under the same co-culture ratio (MSCs: Monocytes 2:1) for 2 hours before efferocytosis was assessed. All groups of MSCs were fluorescent-labelled with 20 ng/mL pH-sensitive fluorescent dye pHrodo™Red. Monocytes undergoing efferocytosis were considered as V450⁺ population acquiring the PE fluorescence (Q2 upper-right quadrant: V450⁺ and PE⁺). (B) Percentage of efferocytosis was determined according to the population of Q2 quadrant. Experimental data were expressed as mean \pm SD, n=4. One-way ANOVA and post-hoc Tukey test were used to compare the mean differences among the samples (** *p*-values < 0.01; ns, not significant).

A Changes of serum PGE₂ in PR



B Changes of serum PGE₂ in NR



Supplementary Figure 3. Change of serum PGE₂ level in responder (A) and non-responder (B) GvHD patients. For each patient, the sera before MSC treatment

(Pre MSCs) and after MSC treatment (Post MSCs) have been examined for the level of PGE₂ using ELISA kit.