In vitro study of the host responses to model biomaterials via a fibroblast/macrophage co-culture system – Supplemental material

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1. Materials and Methods

1.1. Cell culture

Cells of the human monocytic cell line THP-1 (DSMZ, Braunschweig, Germany) were cultured in RPMI-1640 medium (Biochrom AG, Germany) supplemented with 10% (v/v) FBS (Biochrom AG) and 1% (v/v) AAS (Sigma-Aldrich, Germany) at 37 ºC in a humidified 5% CO_2/95% air atmosphere. Suspended cells were split by centrifugation. The old medium was removed and the cell pellet was resuspended in fresh medium every second day in order to maintain a cell density of 0.5-1.0 x 10^6 cells mL^{-1}. The macrophages were obtained by incubation of THP-1 cells with 200 nM phorbol-12-myristate-13-acetate (PMA, Sigma-Aldrich, Germany) for 48 h and another 24 h without PMA.

1.2. Immunofluorescence staining of PMA-induced THP-1 cells

The macrophages were treated and incubated as described above on cover slips (⌀12 mm, Menzel, Germany) at a concentration of 2x10^5 cells per well in 24-well plates. Afterwards, the cells were fixed with 4% (w/v) paraformaldehyde (PFA, Sigma-Aldrich, Germany) in PBS for 15 min and permeabilized with 0.1% (v/v) Triton X-100 (Sigma, Germany) for 10 min. After three times rinsing with PBS, the non-specific binding sites were blocked by incubation with 1% (w/v) bovine serum albumin (BSA, Merk, Germany) for 1 h. Antibodies were diluted in 1% (w/v) BSA in PBS. The cells were incubated with a mouse monoclonal antibody against B7-1 (1:50, Santa Cruz Biotechnology Inc.,

USA), mouse monoclonal antibody against CD163 (1:50, Santa Cruz Biotechnology Inc., USA) or rabbit polyclonal antibody against CD68 (1:50, Santa Cruz Biotechnology Inc., USA) over night at 4°C. Subsequently, after washing with PBS three times, the secondary goat-anti-mouse antibody conjugated with CY2 (1:100, Dianova, Germany) and the goat-anti-rabbit antibody conjugated with CY3 (1:100, Dianova, Germany) were incubated with the cells for 1 h. Nuclei were stained by TO-PRO3 (1:500, Invitrogen, Germany) for 30 min incubation. The samples were then washed, mounted with Mowiol (Calbiochem, Darmstadt, Germany) and examined with confocal laser scanning microscopy (CLSM, LSM 710, Carl Zeiss, Oberkochen, Germany) using a 10x objective. Images were processed with the ZEN2011 software (Carl Zeiss, Oberkochen, Germany).

1.3. Flow cytometry of PMA-induced THP-1 cells

The macrophages were generated and incubated as described above at a concentration of 1.0 x 10^6 cells per flask in T25 cell culture flasks (Greiner Bio-One, Frickenhausen, Germany). Afterwards, the macrophages were incubated in 10 mM EDTA in Ca^{2+}, Mg^{2+} free PBS, placed for 5 min on ice and after 15 min incubation at 37 ºC in a humidified 5% CO_2/95% air atmosphere, the cells were detached with a cell scraper. The detached macrophages were incubated for 30 min at 4°C with 1 µg per 1x10^6 cells of the primary antibody in 3% BSA/PBS. Here a mouse monoclonal antibody against B7-1 (1:50, Santa Cruz Biotechnology Inc., USA), or a mouse monoclonal antibody against CD163 (1:50, Santa Cruz Biotechnology Inc., USA) and a rabbit polyclonal antibody against CD68 (1:50, Santa Cruz Biotechnology Inc., USA) were used. After three times washing the cell solution was incubated for 30 min at 4°C in the dark with the fluorochrome-labeled secondary antibodies, a goat-anti-mouse IgG-FITC and a goat-anti-rabbit IgG-CFL 647 (0.5 µg per 1x10^6 cells, both from Santa Cruz Biotechnology Inc., USA). Finally, the cells were incubated 30 min with a PE conjugated mouse anti-Human CD14 antibody (BD Bioscience, Germany). The controls were incubated with only the secondary antibody, to rule out the staining due to autofluorescence or any unspecific binding during the measurement. Afterwards the cells were fixed with 1% Paraformaldehyde for 15 min and resuspended in 10% FBS, 1% sodium azide in PBS. Measurements were done with flow cytometer LSR II Fortessa using the FACS-Diva software (BD Bioscience, Germany).

2. Results

Immunofluorescence staining and flow cytometry were done to characterize the PMA-induced THP-1 cells regarding their differentiation into macrophages with further lineage development into M1 or M2 phenotypes. Therefore we stained the PMA-induced THP-1 against CD14 as a marker for monocytes, CD68 a marker for M0 macrophages, CD80 for M1 and CD163 for M2 macrophages. In the immunofluorescence images we found after induction with PMA that the majority of the cells was positive for CD68 (Fig.1) and some cells were positive for CD14. However, exposing the cells to CD80 or CD163 antibodies did not show any visible staining, which indicated that PMA treatment leads to an early macrophage phenotype not yet committed to M1 or M2. Since flow cytometry is more sensitive especially for low or weak expression of antigens, we analysed PMA-induced THP-1 cells regarding the expression of the same surface markers as described above. Fig. 2 shows that we found a high number of CD68 positive cells of about 85% (Fig. 2A), but with different intensities of the positive cells. Further we detected also a fraction of cells positive for CD14, but in a lower amount of 31.7% (Fig. 2B). Again, no significant detection of M1 and M2 differentiated macrophages was possible because only a small part of cells was positive for CD80 (0.65%, Fig. 2D) and CD163 (2.05%, Fig. 2C).

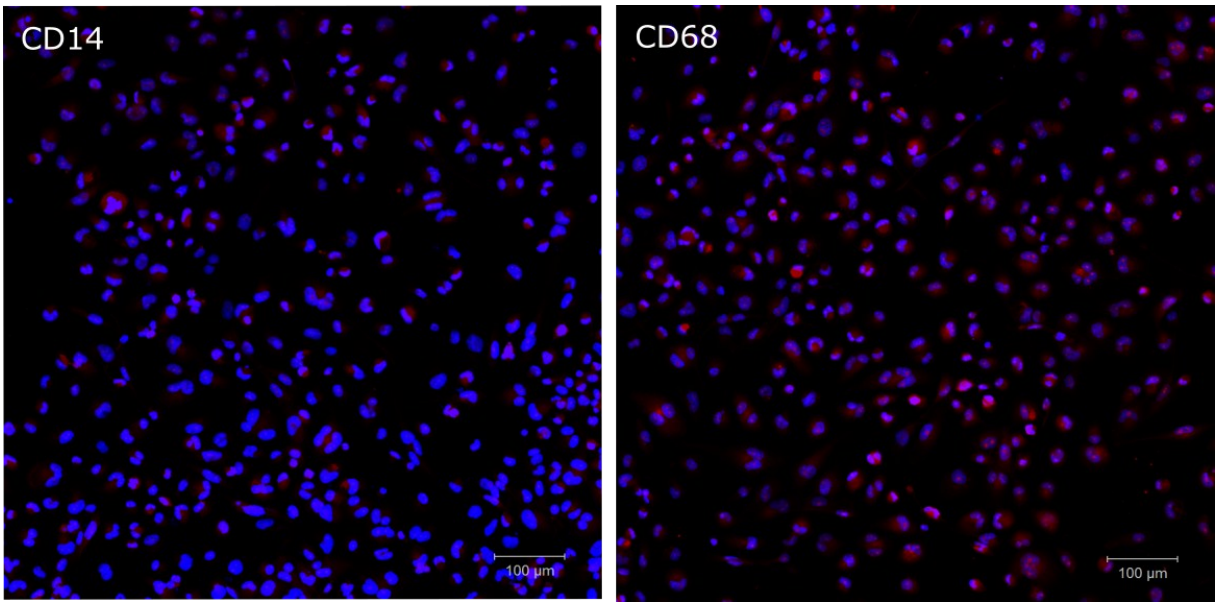

Fig. 1: Immunofluorescence staining of CD14 (red), CD68 (red) and nuclei (blue) after 48 h PMA incubation and another 24 h without PMA. The CD80 and CD163 were not visible under the same conditions. Bar = 100 µm.

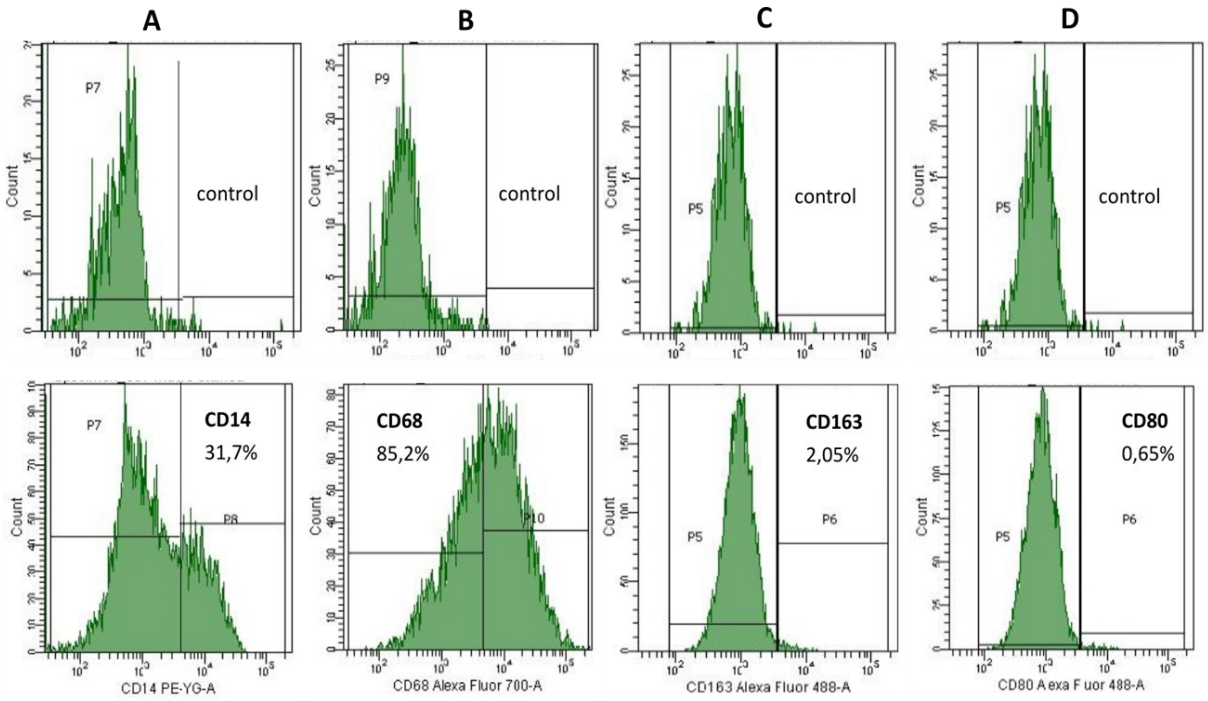

Fig. 2: Cell surface expression of CD14, CD68, CD163 and CD80 of PMA-induced THP-1 cells was analysed by flow cytometry. The first line shows the particular control (A unstained cells, CD14 direct labelled with PE, no secondary antibody; B only secondary antibody goat-anti-rabbit IgG-CFL 647; C and D only secondary antibody goat-anti-mouse IgG-FITC) to the staining with different surface markers in the second line. Data represent mean, n = 3.

3. Conclusion

During treatment with PMA the THP-1 cells stopped proliferation, became adherent and differentiated subsequently to macrophages (CD68 positive).[1] A further characterising of the PMA-induced cells was performed to see if these cells express a M1 (positive for CD80)[2] or M2 (positive for CD163)[3] phenotype. Both technics showed no significant expression of M1 or M2 specific marker. The treatment of the THP-1cells with PMA induced obviously a M0 macrophage phenotype, [2, 3] which can

be further developed to both lineages M1 and M2.[2,4,5] This protocol produces obviously an early phenotype of macrophages positive for CD68, but not polarized phenotypes like M1 or M2 macrophages. The results of the current study fit very well with previous data from Mittar et al., 2011, where no expression of CD80 and CD163 was found after PMA-treatment of THP-1 cells.

4. References

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