Macrophage Phagocytosis and Efferocytosis: Implications for Therapeutic Modulation in the Tumor Microenvironment

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Background

Macrophages play a central role in the tumor microenvironment. Depending on their activation state and local signals, they can exert both pro- and anti-tumorigenic effects. Tumor-associated macrophages (TAMs) often exhibit an immuno-suppressive, pro-tumoral phenotype that promotes tumor growth, angiogenesis, metastasis, and therapy resistance. Due to their plasticity and abundance in solid tumors, understanding TAMs behavior in cancer is critical for developing effective immunotherapies. This requires reliable, physiologically relevant in vitro assays modeling key functions, such as phagocytosis, efferocytosis, cytokine secretion, and tumor cell interaction.

Method

CD14+ monocytes were isolated from cryo-conserved PBMCs, differentiated into M0 macrophages with M-CSF for 6 days. Macrophages were polarized into M1 (IFNγ + LPS) or M2 TAM-like (IL-4 + IL-10 + TGF-β) phenotypes for additional 2 days. M2 repolarization into M1 phenotype was achieved by addition of M1 polarizing cytokines (IFNγ + LPS). Phenotypical characterization was performed by surface staining for viability, CD86, CD163, CD206, CD209 and flow cytometry analysis.

Real-time imaging-based phagocytosis and efferocytosis assays were performed using BioTek Cytation 5 Cell Imaging Multimode Reader (Agilent). Macrophages were seeded in a 384-well Optical Imaging plate, together with either pHrodo™ Red Zymosan BioParticles™ Conjugates (for phagocytosis) or Camptothecin-treated pHrodo™ labelled Raji cells (for efferocytosis). Cytation 5 was programmed to perform high contrast brightfield and fluorescent imaging of the region of interest (ROI, 10x objective) central area of each well for each well every 30-40 minutes over a period of 12-24 hours. Images were preprocessed using a "Rolling Ball" algorithm to increase signal-to-background ratio and subsequently used as input for "Object Analysis". Objects were defined as areas within the ROI having pixel fluorescence intensity above the set threshold and an overall size between 2 and 200 µm. Total Sum Area (µm²) was used as metric of interest for these assays, defined as the sum of the areas of all the objects identified within the ROI.

After 24 hours, supernatants were collected and *pro-inflammatory cytokine quantification* was performed using MSD U-Plex kit (Meso Scale Discovery® (MSD) platform), following manufacturer's instructions.

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CD14+ monocyte differentiation and polarization

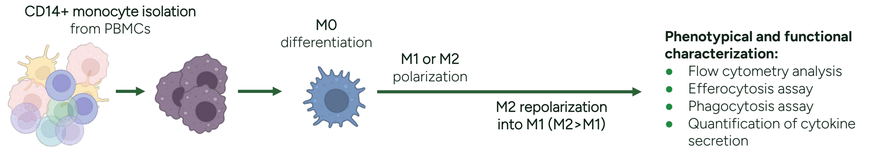

Figure 1: CD14+ monocyte differentiation and polarization. CD14+ monocytes were isolated from cryo-preserved PBMCs. After culture with M-CSF, M0 macrophages were polarized into M1 or M2 phenotypes. Finally, M2 macrophages were re-polarized to M1 ("M2>M1") by adding M1 cytokine polarization cocktail. Efferocytosis and phagocytosis assays, flow cytometry analysis and cytokine secretion quantification were used as main techniques for phenotypical and functional characterization of M0, M1, M2, and M2 repolarized into M1 macrophages (hereby "M2>M1 macrophages").

Phenotypical characterization of key macrophage markers in M0, M1 and M2>M1 macrophages

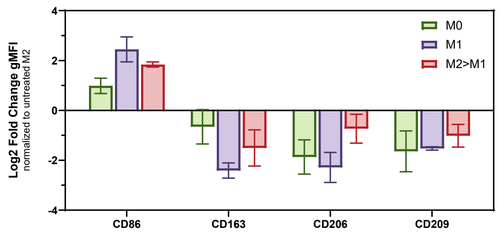

Figure 2: Phenotypical characterization of key macrophage markers in M0, M1 and M2>M1 macrophages. Expression of key surface markers was assessed by flow cytometry analysis. Geometric Mean Fluorescent Intensity (gMFI) in M0, M1 and M2>M1 macrophages was normalized to gMFI in M2 macrophages, to better visualize changes across macrophage types. In M1 and M0 phenotypes, CD163, CD206, CD209 (key M2 markers) expression is reduced while CD86 (key M1 marker) is increased.

Here we show that expression pattern in M2>M1 macrophages resembles M1 phenotype. (mean ± SD, *n*=3).

Real-time imaging-based quantification of engulfment of pHrodo™ labelled apoptotic Raji cells (Efferocytosis), using Agilent Cytation 5 technology

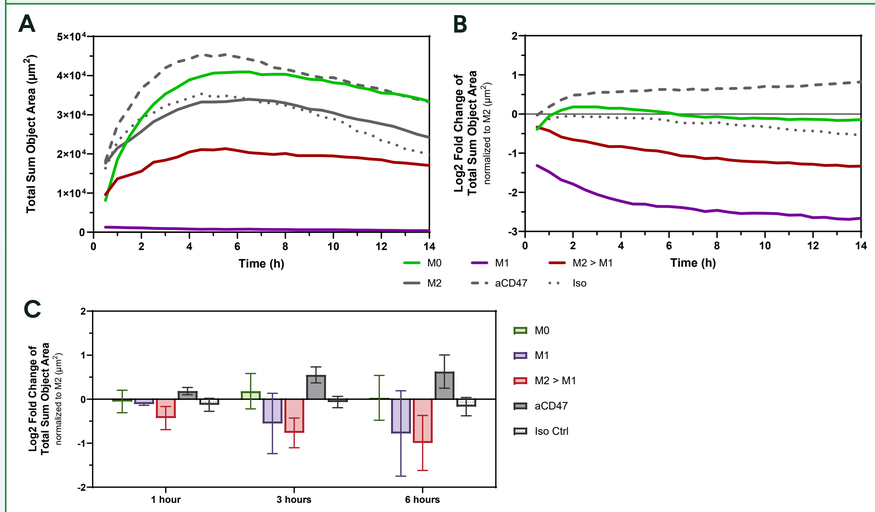

Figure 3: Real-time imaging-based quantification of engulfment of pHrodo™ labelled apoptotic Raji cells (Efferocytosis), using Agilent Cytation 5 technology. Macrophages were incubated with apoptotic pHrodo™ labelled Raji cells for over 12 hours.
(A) Successful uptake and engulfment of apoptotic cells was measured by increased fluorescent area (Total Sum Object Area µm²) over time (representative plot, *n*=1). Here we show that M2 macrophages have the highest uptake rate, which is further increased upon addition of an anti-human CD47 antibody (aCD47, dashed grey line).
(B, C) In line with previous findings, M1 macrophages show minimal uptake, while it is moderately reduced in M2>M1 macrophages compared to M2. (mean ± SD, *n*=3)

aCD47*: anti-human CD47 monoclonal antibody;* iso ctrl*: isotype control antibody*

Real-time imaging-based quantification of phagocytic activity of pHrodo™ Zymosan BioParticles™ conjugates, using Agilent Cytation 5 technology

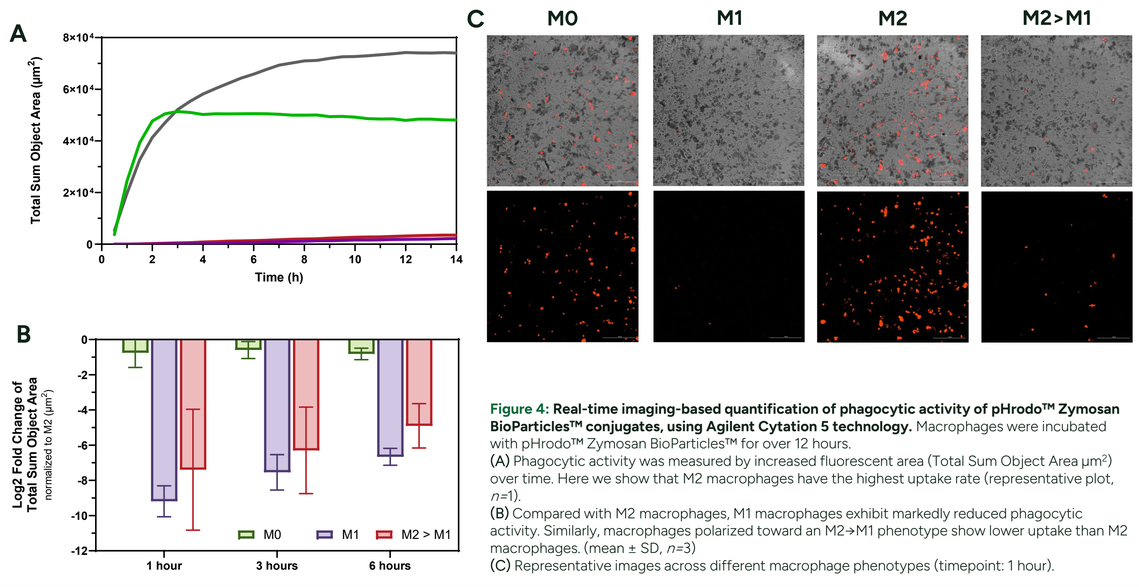

Figure 4: Real-time imaging-based quantification of phagocytic activity of pHrodo™ Zymosan BioParticles™ conjugates, using Agilent Cytation 5 technology. Macrophages were incubated with pHrodo™ Zymosan BioParticles™ for over 12 hours.
(A) Phagocytic activity was measured by increased fluorescent area (Total Sum Object Area µm²) over time. Here we show that M2 macrophages have the highest uptake rate (representative plot, *n*=1).
(B) Compared with M2 macrophages, M1 macrophages exhibit markedly reduced phagocytic activity. Similarly, macrophages polarized toward an M2→M1 phenotype show lower uptake than M2 macrophages. (mean ± SD, *n*=3)
(C) Representative images across different macrophage phenotypes (timepoint: 1 hour).

Quantification of cytokine secretion: comparison between efferocytosis and phagocytosis assay

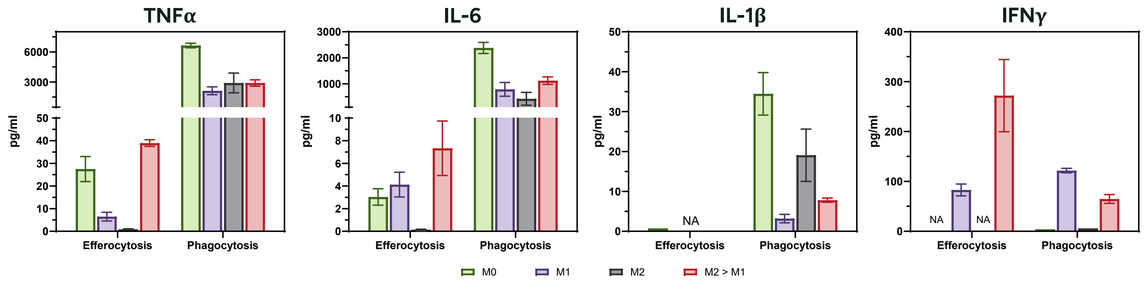

Figure 5: Quantification of cytokine secretion: comparison between efferocytosis and phagocytosis assay. Supernatants were collected 24 hours after the start of efferocytosis and phagocytosis assays, and secretion of pro-inflammatory cytokines (TNFα, IL-6, IL-1β, IFNγ) was evaluated using the Meso Scale Discovery® (MSD) platform. Here, we show that macrophage polarization not only influences phagocytic and efferocytic behavior but also shapes the secretion profile of pro-inflammatory cytokines. As expected, efferocytosis is associated with low pro-inflammatory cytokine secretion, whereas phagocytosis induces high pro-inflammatory cytokine production.

M2-mediated efferocytosis triggers an anti-inflammatory (and pro-tumoral) response, with minimal TNFα, IL-6, IL-1β, IFNγ production. When repolarized to M1, cytokine secretion pattern of M2>M1 macrophages was comparable (if any increased) to M1 macrophages.

Summary

- Repolarization of M2 macrophages to M1 affects key surface marker expression
- M0, M2 and TAM-like macrophages have a strong capacity for phagocytosis and efferocytosis, M1 macrophages have minimal particle or apoptotic tumor cell uptake
- Time-resolved analysis revealed distinct uptake patterns across macrophage phenotypes
- M1-repolarized M2 macrophages showed reduced phagocytic, but not efferocytic, activity
- Efferocytosis does not trigger a pro-inflammatory cytokine response
- Repolarization of M2 macrophages toward an M1 phenotype results in a cytokine expression profile comparable to that of M1 macrophages during both efferocytosis and phagocytosis