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Research paper

Yeast-mediated mRNA delivery polarizes immuno-suppressive macrophages towards an immuno-stimulatory phenotype

Michelle Seif [a,b], Jessica Hoppstädter [b], Frank Breinig [c], Alexandra K. Kiemer [b,*]

[a] Korea Institute of Science and Technology Europe, Saarbruecken, Germany
[b] Department of Pharmacy, Pharmaceutical Biology, Saarland University, Saarbruecken, Germany
[c] Molecular and Cell Biology and Center of Human and Molecular Biology (ZHMB), Saarland University, Saarbruecken, Germany

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ABSTRACT

Macrophages have increasingly gained interest as a therapeutic target since they represent an integral component of the tumor microenvironment. In fact, M2 macrophage accumulation in solid tumors is associated with poor prognosis and therapy failure. Therefore, reprogramming M2 macrophages towards an M1 phenotype with anti-tumor activity by gene therapy represents a promising therapeutic approach. Herein, we describe recombinant *Saccharomyces cerevisiae* as a novel gene delivery vehicle for primary human macrophages. Opsonized *S. cerevisiae* was taken up efficiently by M2 macrophages and initiated the expression of pro-inflammatory cytokines. Recombinant yeast delivered functional nucleic acids to macrophages, especially when constitutively biosynthesized mRNA was used as cargo. Interestingly, expression of the protein encoded for by the delivered nucleic acid was higher in M2 cells when compared to M1 macrophages. Finally, the delivery of mRNA coding for the pro-inflammatory regulators *MYD88* and *TNF* to M2 macrophages induced a prolonged upregulation of pro-inflammatory and cytotoxic cytokines in these cells, suggesting their successful re-education towards an anti-tumor M1 phenotype. Our results suggest the use of yeast-based gene delivery as a promising approach for the treatment of pathologic conditions that may benefit from the presence of M1-polarized macrophages, such as cancer.

1. Introduction

Cell-specific gene delivery represents a promising therapeutic approach, but limited specificity and bioavailability is still a major obstacle [1]. Macrophages as critical players in inflammatory diseases have gained increasing attention as potential targets for both drug [2] and gene delivery strategies. Still, therapeutic approaches mostly aim to target the inflammatory macrophage phenotype [1].

Macrophages are professional phagocytic cells with a high heterogeneity and remarkable plasticity. Their functional phenotype can be dictated by the signals received from their microenvironment. With reference to Th1-Th2 activation of T cells, macrophages with a pro-inflammatory phenotype due to Th1 cytokine or pathogen exposure were termed M1 ("classically activated") and those exposed to Th2 cytokines with enhanced anti-inflammatory functions were referred to as M2 ("alternatively activated") macrophages [3].

The macrophage phenotype can be both protective and pathogenic. Whereas M1 macrophages are essential for pathogen clearance and tumor suppression, they can also contribute to the pathogenesis of autoimmune and chronic inflammatory diseases. The M2 phenotype is critically involved in wound healing but is also known to promote tumor growth [4]. Macrophages are one of the major populations of infiltrating leukocytes in solid tumors. Tumor-associated macrophages (TAM) are considered to be a polarized M2-like macrophage population with potent immunosuppressive functions [5–7]. TAM play a significant role in tumor initiation, development, and metastasis. Accordingly, high numbers of TAM are related to a poor prognosis [8–11] and therapy failure [12].

The plasticity of macrophages provides a basis for strategies aiming to re-educate TAM towards an M1 phenotype. Nucleic acid therapy using adenoviral vectors to increase the expression of pro-inflammatory mediators in macrophages showed promising results [1]. In an *in vitro* model of human M1/M2 polarized macrophages, Krausgruber et al. showed that the forced expression of the M1-related transcription factor interferon regulatory factor 5 (IRF5) in M2 macrophages re-educated them to an M1 phenotype by inducing the expression of pro-inflammatory cytokines and costimulatory molecules [13]. Adenoviral delivery of cytokine

* Corresponding author at: Department of Pharmacy, Pharmaceutical Biology, Saarland University, Campus C2 3, 66123 Saarbruecken, Germany.
E-mail address: pharm.bio.kiemer@mx.uni-saarland.de (A.K. Kiemer).

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DNA, e.g. encoding for interleukin (IL)-12 or interferon (IFN)-γ, was able to induce an M1 phenotype in murine macrophages. Adoptive transfer of these genetically modified macrophages into tumor-bearing mice showed encouraging anti-tumor effects [14–16]. Due to the lack of specificity and potential toxicity of viral vectors, novel non-viral gene delivery systems are required [1].

Recent studies have shown that recombinant yeast, i.e. *S. cerevisiae*, can be used as a vehicle for oral DNA- or RNA-based vaccination [17–20]. Moreover, recombinant *S. cerevisiae* expressing tumor-associated antigens is presently evaluated in clinical trials as a subcutaneous therapeutic anti-cancer vaccine [21,22]. Importantly, *S. cerevisiae* is classified as GRAS (generally recognized as safe) organism. We hypothesized that yeast might be used to target phagocytic cells, such as macrophages, in the context of gene delivery.

In this study, we analyzed the potency of *S. cerevisiae* to deliver nucleic acids to primary human macrophages and determined whether this approach might be used to re-educate M2 macrophages towards an M1 phenotype.

2. Materials and methods

2.1. Preparation and culture of human monocyte-derived macrophages (MDM)

Human peripheral blood monocytes were prepared as described previously [23,24]. Peripheral blood mononuclear cells (PBMC) were obtained from buffy coats by density gradient centrifugation using Pancoll (PAN Biotech, Aidenbach, Germany). Erythrocytes were lysed using BD Pharm Lyse (BD Biosciences, Heidelberg, Germany). PBMC were washed in endotoxin-free PBS (phosphate buffered saline, Sigma-Aldrich, Steinheim, Germany) containing 2 mM EDTA (ethylenediaminetetraacetic acid, Sigma-Aldrich). Monocytes were isolated from PBMC by positive selection using magnetic anti-CD14 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). Monocyte purity was > 95% as assessed by CD14 expression (data not shown).

For macrophage polarization, monocytes were cultured in 12-well plates at a density of 0.5×10^6 cells per well for 5 days at 37 °C and 5% CO_2 in Macrophage-SFM (Life Technologies, Grand Island, NY, USA) supplemented with either 10 ng/ml human recombinant macrophage colony-stimulating factor (M-CSF) or granulocyte-macrophage colony-stimulating factor (GM-CSF) (Miltenyi Biotec). The medium was changed every other day. GM-CSF-differentiated macrophages (GM-MΦ) were stimulated for another 40 h or as indicated with 1 μg/ml LPS (Sigma-Aldrich) and 20 ng/ml human recombinant IFN- γ (Miltenyi Biotec). M-CSF-differentiated macrophages (M-MΦ) were cultured for 40 h or as indicated with 200 ng/ml human recombinant IL-10 (Miltenyi Biotec). All cytokines and growth factors were dissolved in endotoxin-free water (Sigma-Aldrich).

For yeast-macrophage co-culture experiments, monocytes were cultured in Petri dishes (Ø 60 mm) at a density of 6×10^6 cells per dish for 4 days at 37 °C and 5% CO_2 in Macrophage-SFM (Life Technologies) supplemented with GM-CSF or M-CSF as described above. On day 4, cells were harvested using PBS supplemented with 5 mM EDTA (Sigma-Aldrich) and seeded into 12-well plates at a density of 5×10^5 cells/well. On the next day, cells were stimulated with LPS/IFN-γ or IL-10 as described above. In all experiments comparing M1 and M2 macrophages, cells were generated from monocytes obtained from the same donor.

2.2. Yeast transformation, cultivation, and preparation

The generation of plasmids pCMV-IRES-eGFP, pMLS1-IRES-eGFP, pICL1-IRES-eGFP, and pPGK-IRES-eGFP was previously described [18]. The DNA sequence coding for the IRES-TNF or IRES-MyD88 fusion transcript was synthesized by GeneArt (Invitrogen). The IRES-TNF or IRES-MyD88 sequence (supplementary data) was cloned into the pPGK-IRES-eGFP vector after XhoI/BglII digestion, resulting in IRES-TNF or IRES-MyD88 replacing the IRES-eGFP sequence. For cloning, ultracompetent *E. coli* cells (NEB 10-beta competent *E. coli*, New England BioLabs, MA, USA) were grown at 37 °C in LB medium supplemented with 100 μg/ml ampicillin. Transformants were selected, and the plasmid pPGK-IRES-TNF was isolated and sequenced (Eurofins MWG Operon, Ebersberg, Germany). The *S. cerevisiae* strain S86c [MATaura3-2 his3 pra1 prb2 prc1 cps1L-0M-0] was grown overnight at 30 °C in YPD medium (1% yeast extract, 2% peptone, 2% glucose). Yeast cells were transformed by the lithium acetate method [25], and transformants were selected on synthetic complete (SC) medium (0.17% yeast nitrogen base, 0.5% ammonium sulfate, supplemented with amino acids, nucleotides, and 2% glucose) lacking uracil.

For yeast uptake studies, *S. cerevisiae* cells were cultured in SC medium overnight at 30 °C while shaking at 220 rpm. For CFSE (carboxyfluorescein diacetate succinimidyl ester) labeling, 10^7 yeast cells were harvested by centrifugation at $3000 \times g$ for 5 min. After washing twice with endotoxin-free PBS (Sigma-Aldrich), cells were stained with 2.5 μM CFSE (Life Technologies) for 30 min at 37 °C. Cells were washed in PBS containing 5% FBS to remove the residual dye. For nucleic acid delivery experiments, *S. cerevisiae* containing the empty vector (YEp352) or one of the plasmids described above was grown in SC medium lacking uracil in flasks while shaking at 220 rpm at 30 °C until an $O.D._{600}$ of 1 was reached. CFSE-stained yeast cells or recombinant yeast cells were opsonized by incubation with 50% human AB serum (PAA, Pasching, Austria, diluted in PBS), for 30 min at 37 °C. Subsequently, the cells were washed twice with PBS and resuspended in macrophage-SFM medium.

2.3. Expression of surface markers

Macrophages were stained for surface marker analysis by flow cytometry as described [24]. In brief, macrophages were detached from the plates using PBS containing 5 mM EDTA (Sigma-Aldrich). Cells were resuspended in MACS Buffer (PBS pH 7.2, 2 mM EDTA, 0.5% (w/v) BSA, and 0.09% (w/v) NaN_3, Miltenyi Biotec) and blocked using FcR Blocking Reagent (Miltenyi Biotec). Cells were stained with the following antibodies: anti-CD14 (PE, clone TÜK4), anti-CD80 (PE, clone 2D10), anti-HLA-DR, DP, DQ (FITC, clone REA332), anti-CD163 (FITC, clone GHI/61.1), anti-CD206 (APC, clone DCN228) (Miltenyi Biotec), anti-CD86 (PE, clone B7-2), anti-HLA-ABC (FITC, clone W6/32) (eBioscience, San Diego, CA, USA) or corresponding isotype control. Data acquisition was performed on a FACS Calibur (BD Biosciences). Data were analyzed using FlowJo v10 software (FlowJo LLC, Ashland, OR, USA). Results are reported as the relative geometric mean of fluorescence intensity (GMFI; geometric mean of fluorescence intensity of specifically stained cells divided by the geometric mean of fluorescence intensity of corresponding isotype control).

2.4. Loading macrophages with yeast

Macrophages were incubated with CFSE-stained yeast cells (for uptake studies) or with recombinant yeast (for nucleic acids delivery) at a multiplicity of infection (MOI) of 7 according to Seif et al. [24] in a total volume of 1 ml at 37 °C in 5% CO_2 for 4 h (for uptake studies) or as indicated (for nucleic acid delivery). Plates were briefly centrifuged to ensure that yeast and macrophages were in close contact. After incubation, macrophages were washed with PBS and harvested using PBS/EDTA (Sigma-Aldrich). For uptake