

***Biocompatible surfaces and optimized angiogenesis, aiming at
the development of vascularized biohybrid organs***

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1 Introduction

1.1 Biohybrid organs

Biohybrid organs or tissues combine biological components, such as living cells, with synthetic material that provides mechanical support and spatial organization. Their aim is to reproduce organ or tissue functions, offering new perspectives in regenerative medicine (Attanasio et al., 2016), disease modeling (Yong et al., 2025), and drug testing (Yun et al., 2025). Further, they can provide implantable support or replacement devices. Biohybrid organs are engineered to recreate a complex microenvironment with functional fidelity to human organs or tissues. While 2D cell cultures are more cost-effective, they lack predictability by not enabling the reproduction of complex interactions (Kapałczyńska et al., 2018). Cell architecture, chemical gradients and mechanical stress are important parameters that can highly influence cell metabolism and function but are difficult to reproduce in 2D cell culture. Due to these intrinsic properties, biohybrid organs or tissues can represent strong and reliable models, closer to native tissues, enhancing predictive ability for drug discovery or *in vivo* implantation.

One of the main challenges that restricts the size and scalability of biohybrid organs is the vascular network (Dellaquila et al., 2021). The vascular network is a fundamental structure allowing the proper function of tissues by ensuring cellular homeostasis. It controls the delivery of oxygen and nutrients, but also helps the removal of metabolic wastes (Rosano et al., 2009). Its inner barrier is essentially composed of endothelial cells (ECs) that control blood-tissue exchange and can influence the behavior of other cells, including immune cells. In large engineered tissues and biohybrid organs, the presence of a functional vascular network is critical, as oxygen and nutrient diffusion are limited to approximately 200 μm (Jain et al., 2005). Out of this range cells undergo hypoxia and necrosis due to a lack of gas and nutrient exchange (Tang et al., 2020). Therefore, the formation of a dense and functional vascular network is a critical requirement for sustainable cell viability and function in large biohybrid organs (Novosel et al., 2011).

To overcome this challenge a printed vascular network can be provided (Hann et al., 2021; Jordao et al., 2023). This solution provides a fully customizable and adjustable artificial network that supports cell attachment, proliferation, and organization, while maintaining adequate gas and nutrient exchange. By printing the vascular network, it is possible to tailor the network characteristics to the requirements of the model, such as wall thickness,

porosity, pore sizes, surface topography, shape design, etc. In this study, tubular scaffolds are printed by using the 2PP (2-photon polymerization) printing technique. 2PP enables high spatial resolution down to 100 nm which makes it suitable for vascular network printing (Wu et al., 2006; Fischer & Wegener, 2013). The technique uses a low energy laser beam (near infrared), focused into a tight resin volume by objective lenses. Polymerization is only initiated where the photon density exceeds a certain threshold. The low energy laser ensures that two photons are required and absorbed simultaneously by each photoinitiators contained in the photoresin (Jiang et al., 2016). Therefore, polymerization occurs within a very small volume around the laser focus (called voxel), resulting in high-resolution printing. The desired printings are directly written within the photoresin in contrast to classic 3D printing limited to pile up 2D layers. The photoresins are placed on a transparent substrate: indium-tin-oxide (ITO)-coated glass. The substrate provides mechanical support and must have a refraction index close to that of the photoresin (Ganazhapa et al., 2024).

Additionally, the printed artificial vascular network can be coated with EC that will initiate vasculogenesis and angiogenesis (Hann et al., 2021). While vasculogenesis refers to the *in situ* differentiation of EC and tube growth, angiogenesis refers to the sprouting of new blood vessels from pre-existing ones (Patan, 2004). During vasculogenesis, mesoderm-derived hemangioblasts differentiate into immature EC which migrate, align and organize into primitive tubular structures (Patan, 2000). It is mostly orchestrated by growth factors. VEGF for example promotes endothelial differentiation and migration (Casie Chetty et al., 2017), while bFGF promotes proliferation and network stability (Khan et al., 2017). Maturation of these primary structures involves the recruitment of various cell types such as pericytes and smooth muscle cells to form a fully functional vessel (Coulon et al., 2010).

1.2 Biocompatibility and foreign body reaction

Biocompatibility is a large concept, initially used to describe the ability of biological tissues to co-exist with a material without initiating significant responses by the surrounding environment. Titanium and silicone are defined as inert materials (Habal, 1984; H. J. Lee et al., 2025). They are now often used in orthopedic, dental, or in plastic surgery (Kaur & Singh, 2019; Foroushani et al., 2022; Silva et al., 2022). However, the

notion of inertness evolved with time. Biocompatible material must not only be devoid of toxicity, but it must promote biological interactions, including tissue integration and immune modulation (Williams, 2019). Therefore, according to the purpose of the material, biocompatibility is investigated in many ways. Cytocompatibility defines the ability to support and promote cell adhesion, proliferation and viability in general. Meaning that the behavior of cells is at least not impaired by the material, or in the best case, desired functions can be enhanced. The evaluation of cytocompatibility is usually performed with metabolic assays, tests for membrane integrity (such as lactate dehydrogenase (LDH) assay), and cell proliferation assays (Fotakis & Timbrell, 2006). Morphological and cell adhesion analyses are performed by microscopy. Hemocompatibility refers to the compatibility with blood. A hemocompatible material does not induce hemolysis, platelet activation, complement activation or coagulation (Weber et al., 2018). This is particularly of interest for cardiac implants (Nascimbene et al., 2024). Histocompatibility refers to the integration of material within tissues (Spierings & Fleischhauer, 2019). The material does not cause cell necrosis or promotes strong fibrosis of the surrounding tissues. One of the last dimensions of biocompatibility is immunocompatibility. It defines the ability of a material to interact with the immune system without activation an excessive acute or chronic inflammatory response (Anderson & Miller, 1984).

As soon as a material is implanted, plasma proteins from the host rapidly adsorb onto its surface forming a provisional matrix (Anderson et al., 2008). The amount and type of proteins adsorbing to the surface depend on the material properties, such as roughness, wettability, charge, topography, or chemical composition (Rahmati et al., 2020), and already defines the subsequent implant fate (Wilson et al., 2005). Plasma proteins form a provisional matrix usually made of fibrinogen, complement protein and/or immunoglobulins that can activate the coagulation pathway and complement cascade. These reactions release chemoattractive molecules attracting immune cells towards the implant site (Anderson et al., 2008). This phase is called acute inflammation. Depending on the surface chemistry and adsorbed proteins, infiltrating monocytes differentiate into macrophages, which can further polarize towards a continuum of different macrophage subtypes that are characterized by specific surface markers and cellular functions including cytokine secretion. Pro-inflammatory M1 and anti-inflammatory M2 macrophages are at the extremes of this continuum (Rostam et al., 2016). Macrophages

can also be resident macrophages originating from the yolk sac during embryogenesis (Y. Wu & Hirschi, 2021). They are often considered as major players in inflammation. When inflammation lasts, it is called chronic inflammation. During this phase, M1 macrophages can fuse to form foreign body giant cells and release mediators promoting degradation (Sheikh et al., 2015). This often leads to device degradation (especially in case of degradable material) or device failure. When inflammation resolves, granulation tissue is formed, a highly vascularized tissue rich in fibroblasts. At this stage, macrophages display a M2, anti-inflammatory, phenotype and release anti-inflammatory cytokines such as interleukin (IL)-10, as well as growth factors to promote tissue remodeling, vasculogenesis, and angiogenesis. Granulation tissues can also serve as a precursor for encapsulation, where a collagen-rich fibrous capsule forms around the implant. While this process aims to restore homeostasis and marks the end of the FBR, it might jeopardize the function of the implant due to the physical barrier between the implant and the host tissues (Anderson et al., 2008).

1.3 The role of macrophages in inflammation

M1 macrophages play a major role in inflammation and in host defense against infection. They promote the Th1 responses, which are anti-microbial, anti-viral and anti-tumoral (Gordon, 2003). The M1 phenotype is typically induced by LPS, a component of the outer membrane of Gram-negative bacteria, or interferon (IFN)- γ (Arora et al., 2018). M1 macrophages secrete a broad array of pro-inflammatory molecules such as reactive oxygen species (ROS), IL-1 β , IL-6, or tumor necrosis factor (TNF)- α . These molecules are involved in the recruitment and activation of other immune cells (neutrophils, monocytes, lymphocytes) to sustain and amplify the inflammatory response. M1 macrophages typically express high levels of cluster of differentiation (CD) 38 (especially when the polarization is induced by IFN- γ and LPS), CD80, CD83, and CD86. CD38 is a transmembrane glycoprotein correlated with high secretion levels of ROS and pro-inflammatory cytokines such as IL-6 (Amici et al., 2018). Studies on murine bone-marrow-derived macrophages revealed that CD38 is involved in the resolution of inflammation (Peckert-Maier et al., 2023). CD80 and CD86 are co-stimulatory molecules supporting the activation of T cells. Their expression positively correlates with IL-6 secretion (Balbo et al., 2001; Jiménez-Urbe et al., 2019). Human leukocyte antigen

(HLA)-DR is a human MHC class II protein and mediates antigen presentation to T helper cells (Unanue, 1984). It is generally associated with the M1 phenotype, but can also be expressed by M2 macrophages.

In contrast, M2 macrophages quench the inflammation, promote tissue healing, and elicit Th2 (T helper 2 cell) responses (Gordon, 2003). Their polarization can be induced by the presence of IL-4 and IL-13 (Genin et al., 2015). They are characterized by the production of IL-10, TGF- β and VEGF which inhibit the production of pro-inflammatory cytokines and promote fibroblast activation as well as tissue regeneration, including angiogenesis (Jetten et al., 2014). M2 macrophages typically express CD209, CD206 and CD163 surface markers. CD209 and CD206 are both C-type lectin receptors (a type of pattern recognition receptors (PRRs) involved in immune regulation and tissue homeostasis (Nielsen et al., 2020). They recognize mannose-rich structures, promote cell adhesion and the phagocytosis of either apoptotic cells or microbial components. CD163 is a hemoglobin scavenger receptor and helps in dampening pro-inflammatory signals (Nielsen et al., 2020). CD163 has also been associated with high HIF1- α and VEGF-A expression (Guo et al., 2018). M2 macrophages can be further divided into 4 or even more subtypes. M2a and M2b macrophages can activate Th2 immunity, M2c macrophages are promoting tissue repair and remodeling, and M2d are mainly tumor-associated macrophages promoting angiogenesis and clearing apoptotic cells (Shapouri-Moghaddam et al., 2018; Zhang & Sioud, 2023).

Besides being part of the foreign body reaction, inflammation is the first defense mechanism against pathogens and other endogenous or exogenous threats (Medzhitov, 2008). Inflammation is provoked by the innate immune system that provides a fast, but nonspecific response to stimuli (Chaplin, 2010). These stimuli are classified into two categories, pathogen-associated molecular receptors (PAMPs), and damage-associated molecular receptors (DAMPs), and are mainly recognized by PRRs but also other receptors such as purinergic receptors can be involved. In contrast to PAMPs, DAMPs are endogenous signals that are independent of infection and hence activate a “sterile inflammation” (Kawai & Akira, 2009; Chen & Nuñez, 2010). Common DAMPs are ATP, mitochondrial DNA, or heat-shock proteins, while common PAMPs are LPS, or other bacterial components like flagellin (Kono & Rock, 2008). In the biocompatibility context, materials can induce sterile inflammation, even without plasma proteins adsorbing onto the surface of the material (Sun et al., 2013; Ramenzoni et al., 2021). On stiff and

hydrophobic material, macrophage adhesion triggers cytoskeletal reorganization inducing mechanical and redox stress. These macrophages exhibit a high phagocytic capacity and release pro-inflammatory ROS, cytokines, as well as degradative enzymes in order to neutralize their target (Chuang et al., 2020).

There are different types of PRRs to which PAMPs and DAMPs can bind. One of the most prominent families contains 9 toll-like receptors (TLRs). They all have an ectodomain followed by a transmembrane domain and an intracellular toll-interleukin 1 receptor domain. TLR1, 2, 4, 5 and 6 are expressed on the cell surface, while TLR3, 7, 8 and 9 are localized in endolysosomal compartments. All of them can bind to PAMPs but only 2, 4 and 9 bind DAMPs (Fitzgerald & Kagan, 2020). The nucleotide-binding oligomerization domain-like receptors (NLRs) are another important PRR family that can sense diverse pathogens and cellular stress. This super family can be divided into five subfamilies based on the effector domains at the amino (N)-terminus (Kawai & Akira, 2009).

TLR4 was the first PRR discovered in humans (Medzhitov et al., 1997). It is known to recognize LPS that activates a pro-inflammatory signaling cascade (priming step). Through the activation of the transcription factor, nuclear factor (NF)- κ B and mitogen-activated protein kinases (MAPKs), *IL1B* and *IL6* genes are expressed resulting in the formation of pro-IL-1 β and IL-6. IL-6 is directly secreted by the cells, whereas pro-IL-1 β must be cleaved to generate its active form. IL-1 β maturation depends on the activation of inflammasomes, such as NLR family pyrin domain-containing protein 3 (NLRP3), although other inflammasomes can also mediate its cleavage (He et al., 2016). It can be initiated by various danger signal including ATP binding to P2X7 receptor, or nigericin, a pore-forming bacteria toxin. P2X7 receptor activation triggers K⁺ efflux and thereby the assembly of inflammasome components (NLRP3, ASC and pro-caspase-1). The inflammasome assembly leads to the activation of caspase-1, which cleaves pro-IL-1 β into its bioactive form that can be released by the cells. Nigericin also generates a K⁺ efflux triggering inflammasome assembly and hence, results in the release of mature IL-1 β independently from the P2X7 receptor (Muñoz-Planillo et al., 2013; Pelegrin, 2021).

IL-1 β and IL-6 are two functionally important pro-inflammatory cytokines. They are, among others, very important mediators in inflammation. Cytokines in general are small proteins produced by macrophages but also other cell types. They are used for paracrine

or autocrine communication to regulate the immune system. They bind to specific cytokine receptors and induce signal transduction cascades that eventually alter gene expression. IL-1 β and IL-6, mainly promote cell activation, the expression of pro-inflammatory molecules such as cell adhesion molecules, pro-inflammatory cytokines, or chemokines (Tanaka et al., 2014; Boraschi, 2022).

1.4 The role of macrophages in blood vessel formation

Blood vessel formation is divided into two processes: vasculogenesis and angiogenesis. Vasculogenesis describes *de novo* vessel formation, mostly observed during embryogenesis but it can also occur in adults (Tepper et al., 2005). It starts with the differentiation of mesodermal angioblasts into ECs (Schattelman & Awad, 2004). Angiogenesis defines vessel formation from pre-existing ones. Formation of new blood vessel allows tissue regeneration and growth by providing nutrients and gas exchange to surrounding cells. Therefore, vasculogenesis and angiogenesis are triggered by environmental signals indicating the need for vascularization, generally injury or hypoxia (Marti et al., 2000). During hypoxia, the transcription factor HIF-1 α is stabilized and dimerized with HIF-1 β (Masoud & Li, 2015). It induces the expression of angiogenic molecules including VEGF and bFbF (Calvani et al., 2006; Casie Chetty et al., 2017). VEGF is involved in various signaling cascades promoting cell proliferation, survival, migration, and cytoskeleton organization (Pauty et al., 2017). bFbF is up-regulating VEGF. It also plays a major role in extracellular matrix (ECM) remodeling via matrix-metalloproteases, promoting EC migration (Seghezzi et al., 1998; Toschi et al., 2001).

Macrophages have been shown to produce various factors involved in angiogenesis (Sunderkötter et al., 1994). They secrete the protease MMP9 to modify the ECM (Jadhav et al., 2004), but also growth factors such as VEGF or bFbF. It is still unclear which macrophage subtype could help in angiogenesis. M1 macrophages are often considered as anti-angiogenic (Spiller et al., 2014). By contrast, other authors suggest that they initiate vasculogenesis by modifying the ECM and hence promoting EC migration (Witherel et al., 2021). M1 also secrete VEGF and bFGF. M2, and especially M2a, might be involved in maturation as well as the recruitment of pericytes via PDGF, which stabilize the new blood vessel (Li et al., 2021). They could also regulate functions of M1

macrophages by TIMP3, which inhibits the enzymatic activity of MMP9, VEGF and TNF- α release (Spiller et al., 2014; Deryugina & Quigley, 2015; Corliss et al., 2016).

1.5 Aim of this work

The aim of the first part of this study was to assess the biocompatibility and the inflammatory properties of three different printing resins that shall be used for biohybrid organs. THP-1 cell-derived macrophages differentiated into M1-like macrophages were used to study the inflammatory properties of PlasCLEAR, IP-Visio and IP-S. While the first resin is used for digital light printing (DLP), IP-Visio and IP-S are used for 2PP printing. PlasCLEAR is not suited to print vascular networks. It was of interest to print junctions or outer caging for biohybrid organs, which also need to be biocompatible. THP-1 cells are often used to study inflammation *in vitro*. Inflammatory properties of the polymerized resins were assessed by measuring the concentration of the two pro-inflammatory cytokines, IL-1 β and IL-6 by enzyme-linked immunosorbent assay (ELISA) in cell culture supernatants after cultivation of M1-like macrophages on printed surfaces. These experiments were performed in the absence of further pro-inflammatory stimuli as well as in the presence of LPS, an ATP analogue or nigericin. LDH activity was measured to estimate the viability of the cells. Cells were stained to gain insight on cell adhesion and morphology. Even though cytocompatibility of the resins has already been described in the literature, the inflammatory properties of those materials remain unknown. Understanding and controlling the interaction between biomaterials and macrophages is key when designing biohybrid organs.

The second part of this study aimed at an optimization of vasculogenesis and angiogenesis with the overriding goal to develop vascularized biohybrid organs. The tube formation assay using human umbilical vein endothelial cells (HUVECs) on Matrigel is a very common *in vitro* model to study EC differentiation and capillary sprouting. In this assay HUVECs migrate and organize in a network-like structure, which does not display a proper lumen. This experimental model enables the study of the first steps of vessel formation without considering the stabilization of the network. First, the culture conditions of tube formation assays were simulated in monocultures of THP-1 cell-derived M0-like, M1-like and M2-like macrophages to answer the following questions:

- 1) Which cell culture media are suited to co-culture THP-1 cell-derived macrophages and HUVECs?
- 2) Does Matrigel change the properties of THP-1 cell-derived macrophages?
- 3) Do THP-1 cell-derived macrophages change their phenotype upon prolonged cultivation times, typical for tube formation assays? Thereafter, tube formation assays were performed testing THP-1 cell-derived macrophages and HUVECs in co-culture on Matrigel.

2 Materials and methods

2.1 Materials

2.1.1 Cell lines

Item	Provider	Order code
HUVEC	PromoCell, Heidelberg, Germany	#C-12205
THP-1	German Collection of Microorganisms and Cell Cultures	#ACC16

2.1.2 Reagents

Item	Provider	Order code
2-propanol	Merck Sigma-Aldrich, Darmstadt, Germany	#33539
BD Cytofix/Cytoperm™	BD, Franklin Lakes, NJ, USA	#51-2090KZ
BzATP, 2'-(3')-O-(4-Benzoylbenzoyl)Adenosine-5'-Triphosphate	Jena Bioscience, Jena, Germany	#NU-1620
CytoTox 96® Non-Radioactive Cytotoxicity Assay	Promega, Madison, WI, US	#G1780
ddH₂O, Aqua B. Braun	B. Braun, Melsungen, Germany	#0082479E
DuoSet ELISA Ancillary Reagent Kit 2	R&D Systems, Minneapolis, MN USA	#DY008B
ECGM-2, medium ready-to-use	PromoCell	#C-22011
ECGM-2, Kit	PromoCell	#C-22111

EDTA	Merk Sigma-Aldrich	#20-158
Human IL-1β beta DuoSet ELISA	R&D Systems	#DY201
Human IL-6 DuoSet ELISA	R&D System	#DY206
FBS	Capricorn Scientific, Ebsdorfergrund, Germany	#FBS-16A
Giemsa's Azure Eosin Methylene Blue Solution	Merck Sigma-Aldrich	#1.09204
IFN-γ	R&D Systems	#285-IF
IL-4	Preprotec, Thermo Fisher Scientific, Waltham, MA USA	#200-04
IL-13	Preprotec, Thermo Fisher Scientific	#200-13
LPS from E. Coli O26:B6	Merck Sigma-Aldrich	#L2654
LPS from E. Coli O111:B4	Merck Sigma-Aldrich	#LPS25
Matrigel Matrix	Corning	#354230
May-Grünwald's eosin-methylene blue solution modified	Merck Sigma-Aldrich	#1.01424
Nigericin sodium salt, $\geq$ 98%	Merck Sigma-Aldrich	#N7143
PBS, Dulbecco's phosphate buffered saline	Merck Sigma-Aldrich	#D8537
Penicillin/streptomycin solution (100 X)	Capricorn Scientific	#PS-B
PMA, Phorbol-12-Mystirate-13-Acetate	Merck Sigma-Aldrich	#P1585
RPMI-1640	Capricorn Scientific	#RPMI-A

Trypan blue solution	Merk Sigma-Aldrich	#T8154-100ML
TrypLE solution	Gibco, Thermo Fisher Scientific	#12605-010
Trypsin-EDTA (0.25%) solution in HBSS (1 X)	Capricorn Scientific	#TRY-3B

2.1.3 Printing resins

Resin	Provider
IP-S	Nanoscribe, Karlsruhe, Germany
IP-Visio	Nanoscribe
PlasCLEAR	Asiga, Erfurt, Germany

2.1.4 Antibodies

Antigen	Host	Clone	Ig class	Labelling	Provider, order code
Human CD38	Mouse	HIT2	IgG1, κ	PE-Cy7	BioLegend, #980312
Human CD80	Mouse	BB1	IgM, κ	FITC	BD Pharmingen, #555683
Human CD83	Mouse	HB15e	IgG1, κ	APC	BD, #334098
Human CD86	Mouse	FUN-1	IgG1, κ	PE	BD, #334098
Human CD163	Mouse	GIH/61	IgG1, κ	FITC	BioLegend, # 333617
Human CD206	Mouse	19.2	IgG1, κ	PE	BD Pharmingen, #555954
Human CD209	Mouse	DCN46	IgG2a, κ	PerCP-Cy5.5	BD #334098
Human HLA-DR	Mouse	L243	IgG2a, κ	PerCP-Cy5.5	BD, #339216

2.1.5 Consumables

Item	Provider	Order code
10-well chamber-view slide	Greiner Bio-One, Kremsmünster, Germany	#543078
12-wells cell culture plate	Greiner Bio-One	#665180
48-wells cell culture plate	Greiner Bio-One	#677180
96-wells cell culture plate	Greiner Bio-One	#655 180
Cell culture flask, T-75, surface: standard, filter cap	Sarstedt, Nümbrecht, Germany	#83.3911.002
Cell culture flask, T-25, surface: standard, filter cap	Sarstedt	#83.3910.002
Cell culture flask, T-75, surface: suspension, filter cap	Sarstedt	#83.3911.502
FILTER TIPS 10 µl, 200 µl, 1000 µl	Nerbe plus, Winsen, Germany	#06-613-5300, #06-642-5300, #06-695-5300
Microscope slide	Langenbrinck, Emmendingen, Germany	#03-0004
Pipettes 5 ml, 10 ml, 25 ml	Greiner Bio-One	#606180, #607180N, #760180
PIPETTE TIPS 10 µl, 200 µl, 1000 µl	Sarstedt	#70.1114, #70.3030.020, #70.3050.020
Reaction tubes 0.5 ml, 1.5 ml	Sarstedt	#72.704.400, #72.706
Tubes 15 ml, 50 ml	Greiner Bio-One	#188 271, #237261-N

2.1.6 Equipment and software

Item	Provider
BD FACSLytic™ Flow Cytometer System	BD Biosciences
Biosafety cabinet MSC-Advantage	Thermo Fisher Scientific
Centrifuge, Rotina 420R	Andreas Hettich, Tuttlingen, Germany
CO₂ incubator, Heracell 240i	Thermo Fisher Scientific
Counting chamber, Neubauer, improved	Laboroptik, Lancing, United Kingdom
Inkscape vector graphics edition 1.3.2	Free software distributed under the GNU General Public License, version 3
Microscope Axio Observer	Carl Zeiss Microscopy, Jena, Germany
Microscope camera, Axiocam 305 color	Carl Zeiss Microscopy
Microscope Olympus BX51	Olympus Corporation, Tokyo, Japan
Pipette, Pipetus®	Hirschmann Laborgeräte, Eberstadt, Germany
Pipettes 1 – 10 µl, 10 – 100 µl, 100 – 1000 µl	Eppendorf, Hamburg, Germany
SPSS statistic software version 27.0.0.0	IBM, Ehningen, Germany
Water bath	Köttermann, Uetze, Germany
Zen (blue edition), software	Carl Zeiss Microscopy

2.2 Methods

2.2.1 Media

In this study, different media were used, which base on RPMI-1640 or endothelial cell growth medium (ECGM)-2. Their compositions differ depending on the purpose of the experiments and are abbreviated as follows:

- R: RPMI-1640 + 10% fetal bovine serum (FBS) from Capricorn.
- E: ECGM-2 + 2% FBS + supplements composed of 5 ng/ml EGF + 10 ng/ml bFGF + 20 ng/ml IGF + 0.5 ng/ml VEGF + 1 µg/ml ascorbic acid + 22.5 µg/ml heparin, 0.2 µg/ml hydrocortisone. FBS and supplements were provided by the “ready-to-use” or “kit” pack.
- bE (basal ECGM): ECGM-2 + 2% FBS
- ECGM-2: medium without FBS or supplements.

2.2.2 THP-1 cells

THP-1 cells are a human cell line that originated from an acute monocytic leukemia (Tsuchiya et al., 1980). Once differentiated into macrophages, they were used for assessing cytokine release, or effects on HUVECs. THP-1 cells were grown in suspension in R. The culture was kept at 37°C, 5% CO₂ saturation in a humidified incubator and passaged twice a week. For passaging, the cells were spun down at 500 x g for 8 minutes at room temperature (RT) and resuspended in 5 ml R + 50 U penicillin/ml and 50 µg streptomycin/ml (PenStrep). Cells were counted after adding trypan blue solution (1:10) and 4 x 10⁶ of them were transferred into a new T-75 suspension cell culture flask. Trypan blue stock solution was diluted 1:2 in PBS for every counting.

2.2.3 HUVECs

HUVECs are adherent primary venous EC originating from human umbilical cords. They grow as a monolayer in E. HUVECs were passaged twice a week, when they were approximately 80% confluent and used until passage 5. Between 10,000 and 5,000 cells per cm² were seeded. In T-75 standard cell culture flasks, 2.5 ml 0.25% trypsin-EDTA

was used to detach cells from the surface. The reaction was stopped with 20 ml R. The floating cells were transferred into a 50 ml conical tube for centrifugation for 5 minutes at 300 x g, RT. Finally, the supernatant was discarded, and the pellet was resuspended in 1 ml E for cell counting in 1:10 diluted trypan blue solution.

2.2.4 Differentiation of THP-1 cells towards macrophages

The differentiation of THP-1 cells into macrophages (either M1- or M2-like macrophages) required five days (Figure 1). On the first day, THP-1 cells were spun down at 500 x g, 8 minutes, RT. The pellet was resuspended in 10 ml R + PenStrep. Cells were counted and 6×10^6 cells were transferred into a T75 standard culture flask containing 100 nM phorbol 12-mistirate 13-acetate (PMA) in R + PenStrep to differentiate the cells into M0-like macrophages. The new culture was then incubated at 37°C with 5% CO₂ saturation for 24 hours. The next day, the cells were adherent and were washed with 10 ml R + PenStrep. 20 ml of the same medium was then added, and the culture was placed in the incubator for another 24 hours. On the third day, M0-like macrophages were either exposed to stimuli to polarize them toward M1- or M2-like macrophages or kept as M0-like macrophages by replacing the culture medium with fresh R + PenStrep. The medium in the flask was discarded and replaced by 20 ml fresh medium (R + PenStrep) supplemented with 10 ng/ml IFN γ and 10 ng/ml LPS (*E. coli* 011:B4) for the differentiation towards M1-like macrophages, or 20 ng/ml IL-4 and IL-13 for M2-like macrophages. This time, the culture was placed in the incubator for 48 hours. On the fifth day, the cells were fully differentiated and polarized. They were washed with 10 ml PBS and detached with 5 ml TrypLE. The reaction was stopped after 6 to 8 minutes, when all the cells were floating, by adding 1 ml PBS. They were centrifuged at 100 x g, RT, for 7 minutes and resuspended in 1 ml R + PenStrep to be counted. 50,000 cells were added per well in 200 μ l in a 96-well plate, or in polydimethyl-siloxane (PDMS) wells.

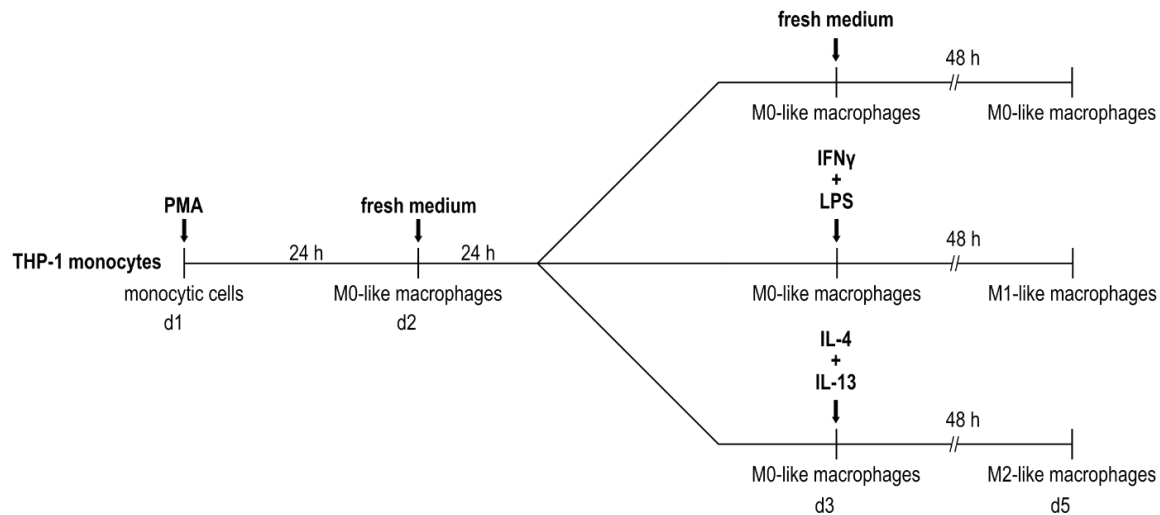


Figure 1: Differentiation of M0-/M1-/M2-like macrophages from THP-1 monocytic cells. IFN, interferon; IL, interleukin; LPS, lipopolysaccharide; PMA, phorbol 12-myristate-13 acetate.

2.2.5 Stimulation of macrophages

Macrophages must be stimulated two times to release IL-1 β . Two experimental set-ups were used depending on the experiment requirements. The first step was to prime cells with 1 μ g/ml LPS (*E. coli* O26:B6). For short-term culture, cells were treated with 1 μ g/ml LPS for 5 hours and then with 200 μ M 2'(3')-O-(4-benzoylbenzoyl)adenosine-5'-triphosphate (BzATP) or 50 μ M of nigericin for 40 minutes (Figure 2A). For longer-term culture, the priming step with LPS started after 14 hours and 20 minutes incubation with fresh medium (Figure 2B). When the experiment was done in a classic well plate, the plate was centrifuged at 500 x g for 8 minutes at 4°C before harvesting the supernatant and storing it at -20°C. When the cells were stimulated in PDMS wells, the supernatant was first collected, followed by centrifugation under the same conditions. Thereafter, the cell-free supernatants (approximately 165 μ l) were frozen at -20°C for future ELISAs.

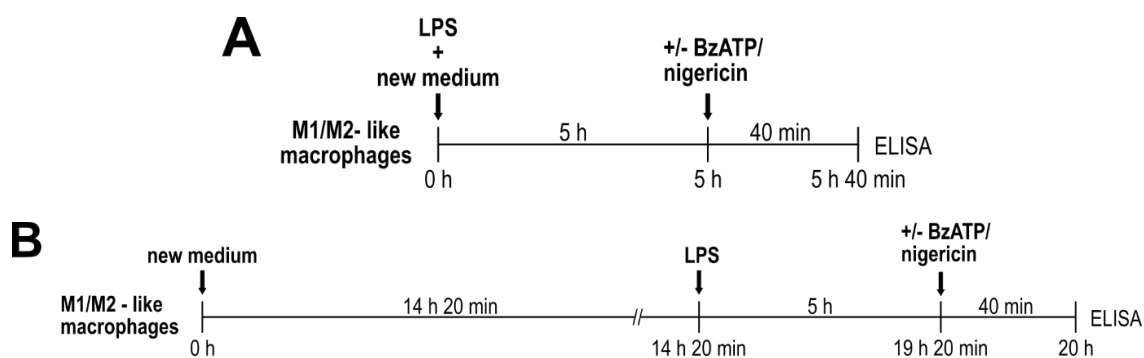


Figure 2: Cytokine release experiment for 6 (A) or 20 hours (B). BzATP, 2'(3')-O-(4-benzoylbenzoyl)adenosine-5'-triphosphate; ELISA, enzyme-linked immunosorbent assay; LPS, lipopolysaccharide

Every IL-1 β release experiment included at least six conditions: control without further stimuli, BzATP, nigericin, LPS, LPS + BzATP, LPS + nigericin. Thus, the effect of the compounds was assessed individually and in combinations of LPS with a second stimulus.

2.2.6 ELISA

The DuoSet Human IL-1 β /IL-1F2 ELISA kit was used in addition to the DuoSet ELISA ancillary reagent kit 2 to quantify IL-1 β in cell-free supernatants. These two kits contained all the solutions mentioned in the following. Assays were performed according to the manufacturer's instructions. 100 μ l capture antibodies diluted in PBS were first pipetted on a 96-well plate and incubated overnight, RT. Wells were washed three times with 400 μ l wash buffer (all wash steps were done with the same volume of wash buffer). Plates were blocked with 300 μ l reagent diluent for an hour, RT, and washed again two times. 100 μ l samples or standards were added for 2 hours, RT. After three washes, 100 μ l of the biotinylated detection antibodies diluted in reagent diluent were added. Plates were covered with an adhesive strip for 2 hours, RT, before being washed three times. 100 μ l Streptavidin-horseradish peroxidase (HRP) solution were added for 20 minutes, and wells were washed three times. After the addition of 100 μ l substrate solution for 20 minutes, RT (avoiding direct light), the reaction was stopped by 50 μ l stop solution.

A standard curve was generated using known concentrations of recombinant human IL-1 β , with a detection range typically spanning 3.91 to 250 pg/ml. Optical density (OD) was measured by using the Fluostar Optima photometer at 450 nm. The OD from the reagent diluent was subtracted from the OD of the samples. The IL-1 β concentration was calculated from the OD data by using the four-parameter logistic (4-PL) curve-fit.

2.2.7 Tube formation assay

To evaluate the angiogenesis of HUVECs, cells were cultivated on Matrigel for 16 to 18 hours. Aliquots of Matrigel were stored at -20°C. The Matrigel was thawed overnight at 4°C and 50 to 60 μ l were pipetted into 96-well plates. Plates were placed into the incubator for an hour for gel formation before adding 100 μ l cell suspension (10,000 cells). In the case of co-culture experiments, 5,000 macrophages (M0-, M1- or M2-like macrophages) were added one hour after the HUVECs. The total HUVEC incubation time for tube formation was 18 hours.

The network quality was assessed by manually counting meshes and segments. A mesh was defined as a closed structure delimited by segments and was only counted when entirely visible within the image. A segment was defined as a linear structure that may occur independently or connected with other segments to form a mesh. Every condition was done in duplicates, and 4 photos were taken from each well. The average of meshes and segments was obtained from the 8 photos of the duplicates and counted as one value for the condition. To limit bias, counting was performed blinded to the experimental conditions.

2.2.8 Flow cytometry

Flow cytometry was performed to evaluate the phenotype of macrophages by their surface markers. Macrophages were first detached from the culture flask with TrypLE (2.5 ml in T-75 flasks) for 7 minutes (37°C). 9 ml of PBS were added to stop the reaction, and cells were centrifuged at 100 x g, RT, 7 minutes. After discarding the supernatant, the cell pellet was resuspended in 0.4 ml flow cytometry buffer (PBS, 0.5 mol/l EDTA, 5% FBS) and counted with trypan blue. Two panels of antibodies against cell surface markers were used (Table 1). Each sample was equally divided into two and centrifuged at 500 x g, RT, 5

minutes. After discarding the supernatant, each sample received the one or the other panel of antibodies (15 μ l for panel 1 and 12 μ l for panel 2) for 30 minutes at 4°C. 1 ml flow cytometry buffer was added and the samples were centrifuged (500 x g, RT, 5 minutes) before discarding the supernatant. Between 200 and 300 μ l FACS buffer was added depending on the expected cell number.

Cells were analyzed using a BD FACSLytic™ Flow Cytometry System (BD Biosciences) and the BD FACSuite™ software, recording at least 10,000 events for each sample. Samples were analyzed using the FlowJo software version 10.8.1 (FlowJo, LLC). Macrophages were first gated by forward scatter-area (FSC-A) and side scatter (SSC)-A, followed by single cell gating by FSC-height (H) and FSC-A.

Table 1: Panel composition

	Antibodies or dye	Volume (μ l per sample)
Panel 1	Anti-CD80	5
	Anti-CD86/-CD209/-CD83	6
	Anti-CD38	2
	DAPI (1:10 in flow cytometry buffer)	2
Panel 2	Anti-CD163	2
	Anti-CD206	3
	Anti-HLA-DR	3
	Anti-CD38	2
	DAPI (1:10 in flow cytometry buffer)	2

CD, cluster of differentiation; DAPI, 4',6-diamidino-2-phenylindole; HLA-DR, human leukocyte antigen-DR

2.2.8 Estimation of cell death

To estimate cell viability, the activity of LDH was measured in cell culture supernatants at the end of the experiments along with IL-1 β quantification by ELISA. The assay was performed according to the manufacturer's instructions using the CytoTox96® Non-

Radioactive, which quantifies LDH activity. 50 µl CytoTox96® Reagent were added to 50 µl sample for 30 minutes, RT. 50 µl stop solution that was included in the kit were then added and the absorbance was measured at 490 nm using the Epoch microplate reader. Background absorbance from R or R + nigericin was subtracted from the values of all samples. LDH activity was expressed as a percentage relative to lysed control cells, which were set to 100%.

2.2.9 Pappenheim staining

Adherent cells were first washed with PBS and then fixed with BD Cytofix/Cytoperm™ solution on ice for 20 minutes. Cells were then washed again with PBS, air-dried and kept at 4°C for a few days before staining or stained immediately. The staining required three steps. First, cells were immersed in May-Grünwald solution for one minute. They were then washed for 5 minutes with distilled water. As a final step, Giemsa's (1:20 diluted in distilled water) solution was added to the cells for 4 minutes. Cells were carefully washed, air-dried.

2.2.10 Preparation of printed surfaces

Five surfaces were used for cytokine release experiments: PlasCLEAR, IP-Visio, IP-S, ITO-coated glass, and glass. IP-Visio, IP-S and PlasCLEAR were 3D-printed. The printing was performed at the Institute of Printing Science and Technology, TU Darmstadt. IP-Visio and IP-S materials were printed by two-photon stereolithography (TPS) with a Nanoscribe Photonic Professional GT2 system (Nanoscribe, Karlsruhe, Germany). PlasCLEAR materials were printed by DLP using an Asiga MAX X 27 system (Asiga, Erfurt, Germany). Glass was a common microscope slide (soda lime glass without surface treatment). The day before cytokine release experiments, all surfaces except PlasCLEAR, were mounted together with PDMS rings forming 4 wells (similar in size to 96-well plates). PDMS rings were held tightly by a washer on the top of the ring and 4 springs on every side to apply even pressure (Figure 3). These devices were tested for tightness with distilled water. All the materials (PDMS wells, washer, springs) were autoclaved before use, except the surfaces (IP-Visio, ITO, glass) that were placed under

UV light in a biological safety cabinet for 30 minutes. PlasCLEAR were placed in 48-well plates.

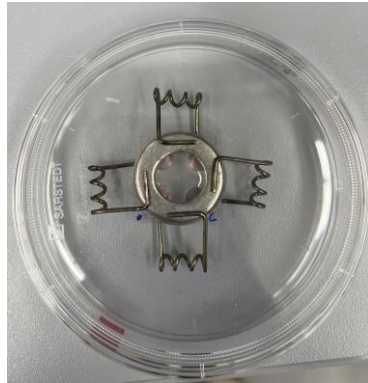


Figure 3: Culture well. Polydimethyl-siloxane (PDMS) culture wells are built on top of printed surfaces.

3 Results

3.1 Immuno- and cytocompatibility of printed surfaces

3.1.1 PlasCLEAR

After the differentiation of monocytic THP-1 cells towards M0-like macrophages and their polarization towards M1-like macrophages, cells were primed with 1 µg/ml LPS to trigger the formation of pro-IL-1 β and IL-6. While IL-6 was released immediately after priming, the maturation and release of IL-1 β depended on a second stimulus, either 200 µM BzATP or 50 µM nigericin (Figure 2A). The release of these two cytokines was evaluated in wells (from 48-well plates) either containing a PlasCLEAR-printed coin or left empty as control. Within each of these two conditions, cells were exposed to six different conditions: no treatment, only LPS, only BzATP, only nigericin, LPS + BzATP, LPS + nigericin.

Independent of the presence of PlasCLEAR, IL-1 β concentrations remained low, when no or one of the stimuli was present (21-272 pg/ml, Figure 4A), whereas the presence of LPS led to an increase in IL-6 concentrations (0-12 pg/ml without LPS, 200-638 pg/ml with LPS, Figure 4B). IL-1 β concentrations considerably increased with the addition of BzATP on macrophages treated with LPS (127-1557 pg/ml, Figure 4A). IL-1 β concentrations in wells containing a PlasCLEAR coin were significantly lower than without (118-1049 pg/ml, $p = 0.028$). After the addition of nigericin to macrophages already treated with LPS, PlasCLEAR also led to a significant reduction in IL-1 β concentrations (198-474 pg/ml without PlasCLEAR, 72-331 pg/ml with PlasCLEAR, $p = 0.028$). This effect was not found for IL-6 ($p < 0.345$); Figure 4B).

Measuring the LDH activity in the supernatants allowed estimation of cell death across all conditions. LDH activities remained between 4 and 28%, regardless of the presence of PlasCLEAR (Figure 4C). Only a single cell passage showed 100% LDH activity with PlasCLEAR and when treated with LPS + nigericin. When LPS and BzATP were combined, PlasCLEAR induced a slight but significant increase in LDH activity ($p = 0.027$).

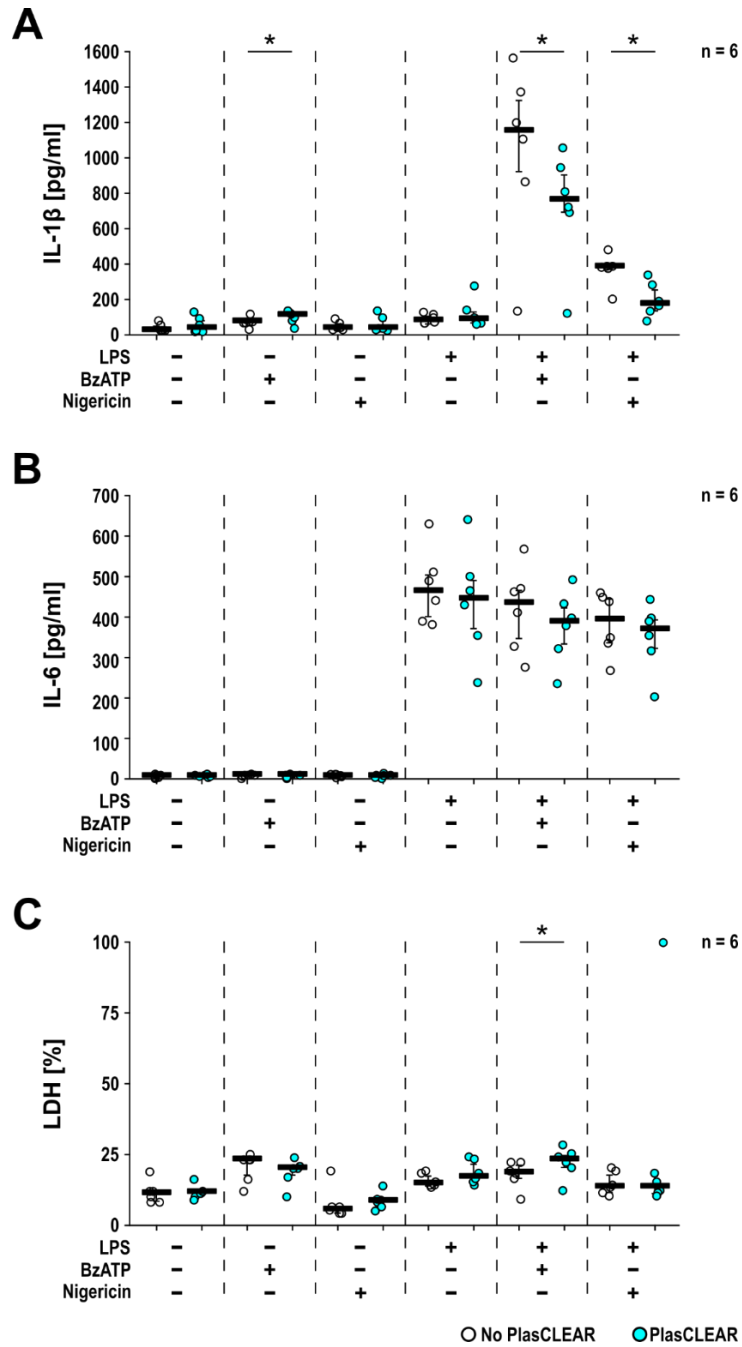


Figure 4: Cytokine concentrations and lactate dehydrogenase (LDH) activity in supernatants of THP-1 cell-derived M1-like macrophages on PlasCLEAR-printed surfaces. Cells were left either untreated or primed with 1 μ g/ml lipopolysaccharide (LPS) for 5 hours. After priming, 200 μ M 2'(3')-O-(4-benzoylbenzoyl)adenosine-5'-triphosphate (BzATP) or 50 μ M nigericin were applied for 40 minutes. (A) IL-1 β concentration, (B) IL-6 concentration, (C) LDH activity. Each dot represents an individual value and bars show the median. Whiskers correspond to the 25th and 75th percentiles, n = 6, * p $\leq$ 0.05. Statistical analysis was performed using the Friedman test, followed by Wilcoxon signed-rank test for pairwise comparisons.

3.1.2 IP-Visio

Pappenheim staining was performed to visualize cell morphology and spread over three distinct surfaces: glass, IP-Visio, and ITO-coated glass. The staining procedure was repeated for each experiment and therefore for each cell passage ($n = 6$). While the IP-Visio surface was slightly uneven due to stitches originating from the printing process, M1-like macrophages neither gathered alongside the stitches nor avoided them. The cell shape and spread were similar across the different surfaces after 6 hours of culture (Figure 5).

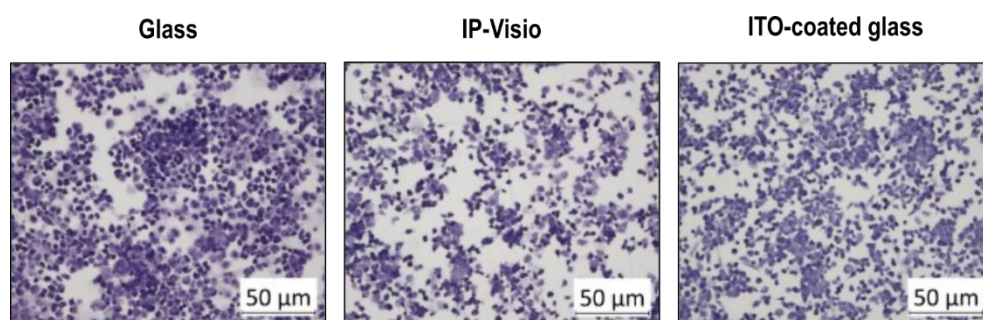


Figure 5: THP-1 cell-derived M1-like macrophages, Pappenheim staining on glass, IP-Visio and indium tin oxide (ITO)-coated glass. 50,000 cells per PDMS well were cultured on different surfaces for 6 hours. They were fixed and stained with Pappenheim staining. One representative passage out of six is shown.

IL-1 β and IL-6 concentrations were evaluated after stimulation of the cells on three different surfaces: glass, IP-Visio and ITO-coated glass. The protocol of the cytokine release experiments is described in Figure 2A). Uncoated glass was the control surface, and the cytokine release from macrophages cultivated on this surface was compared to the one on IP-Visio and on ITO-coated glass.

On all surfaces investigated, IL-1 β concentrations in cell culture supernatants remained low, when none or only one stimulus was present (13-1351 pg/ml, Figure 6A). The same was observed for the IL-6 concentrations. Without LPS, IL-6 concentrations remained low (0-130 pg/ml), independent of the culture surface (Figure 6B). The addition of BzATP on macrophages treated with LPS led to an increased concentration of IL-1 β in the supernatants (948-3449 pg/ml for glass, 1063-5613 pg/ml for IP-Visio, and 947-4534 pg/ml for ITO-coated glass). However, there were no significant differences between the

concentrations on the three culture surfaces (glass *versus* IP-Visio, $p = 0.917$; glass *versus* ITO-coated glass, $p = 0.080$).

The addition of nigericin to macrophages treated with LPS did not trigger a high IL-1 β concentration in contrast to BzATP (111-834 pg/ml on glass, 310-2711 pg/ml on IP-Visio, 166-1724 pg/ml on ITO-coated glass; Figure 6A). These concentrations were similar to the ones of macrophages treated with LPS alone (181-1194 pg/ml for every surface). There was no significant difference among IL-1 β concentrations measured on every surface.

After stimulation of the macrophages with LPS, the IL-6 concentrations were significantly higher on IP-Visio compared to glass (33-385 pg/ml on glass, 141-482 on IP-Visio, $p = 0.043$). The concentrations on ITO-coated glass were not significantly different from glass (61-375 pg/ml, $p = 0.686$; Figure 6B).

All LDH activity values were between 0 and 51% of the total LDH activity but no statistically significant differences were observed between glass, IP-Visio, and ITO-coated glass under the same conditions (Figure 6C).

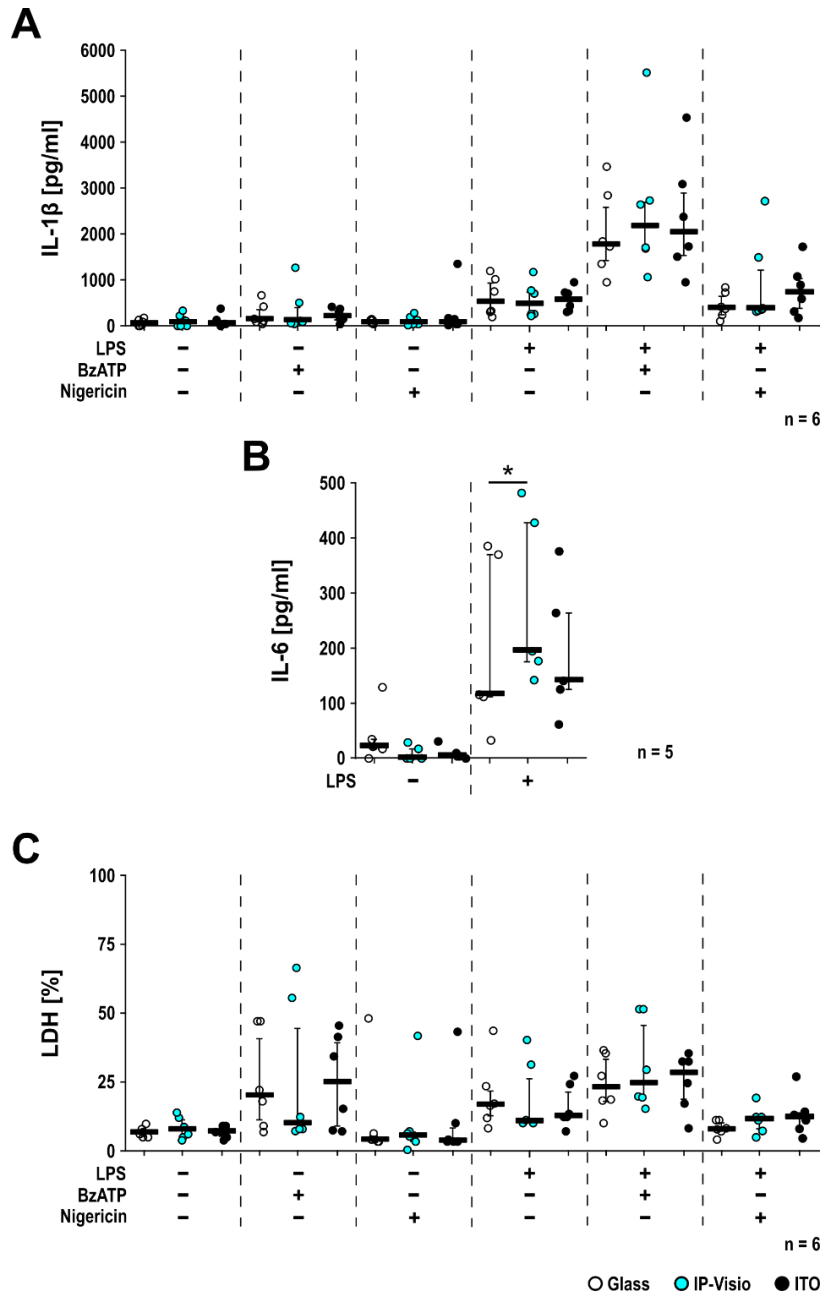


Figure 6: Cytokine concentrations and lactate dehydrogenase (LDH) activity in supernatants of THP-1 cell-derived M1-like macrophages on IP-Visio-printed surfaces, compared to glass and indium tin oxide (ITO)-coated glass. Cells were left either untreated or stimulated with 1 μ g/ml lipopolysaccharide (LPS) for 5 hours. After priming, 200 μ M 2'(3')-O-(4-benzoylbenzoyl)adenosine-5'-triphosphate (BzATP) or 50 μ M nigericin were applied for 40 minutes. (A) IL-1 β concentration, (B) IL-6 concentration, (C) LDH activity. Each dot represents an individual value and bars show the median. Whiskers correspond to the 25th and 75th percentiles, n = 5-6, * $p \leq 0.05$. Statistical analysis was performed using the Friedman test, followed by Wilcoxon signed-rank test for pairwise comparisons.

3.1.3 IP-S

M1-like macrophages on glass and IP-S were stained with Pappenheim staining to analyze their morphology and their adherence as done before with IP-Visio. Again, the spread of the cells was even, despite IP-S stitches (Figure 7). The same experiment as the one performed for IP-Visio was done for IP-S. Measurements on ITO-coated glass were not repeated.

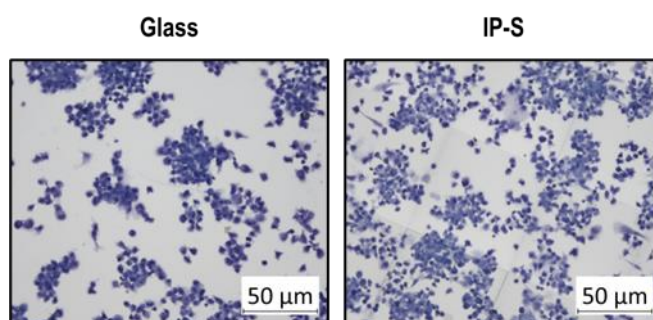


Figure 7: THP-1 cell-derived M1-like macrophages, Pappenheim staining on glass and IP-S. 50,000 cells per polydimethylsiloxane well were cultured on different surfaces for 6 hours. They were fixed and stained with Pappenheim staining. One representative passage out of six is shown.

As long as the cells were treated with only one stimulus, IL-1 β concentrations in cell culture supernatants remained low (6-345 pg/ml, Figure 8A). They increased when macrophages were treated with both LPS and BzATP (1574-3858 pg/ml on glass, 1470-6007 pg/ml on IP-S, $p = 0.463$). When the LPS-primed cells were treated with nigericin instead of BzATP, the IL-1 β concentrations on IP-S were lower than those on glass (145-3163 pg/ml on glass, 76-269 pg/ml on IP-S, $p = 0.046$). This lowering effect was not found in IL-6 concentrations (Figure 8B). Supernatants of untreated cells contained only low concentrations of IL-6 as expected (3-29 pg/ml on glass and IP-S), and their concentrations on glass and IP-S were similar when LPS was added (54-200 pg/ml on glass, 62-211 on IP-S, $p = 0.345$).

The LDH activities consistently suggested a surface-independent cell death (2-33% LDH activity, $p \geq 0.144$, Figure 8).

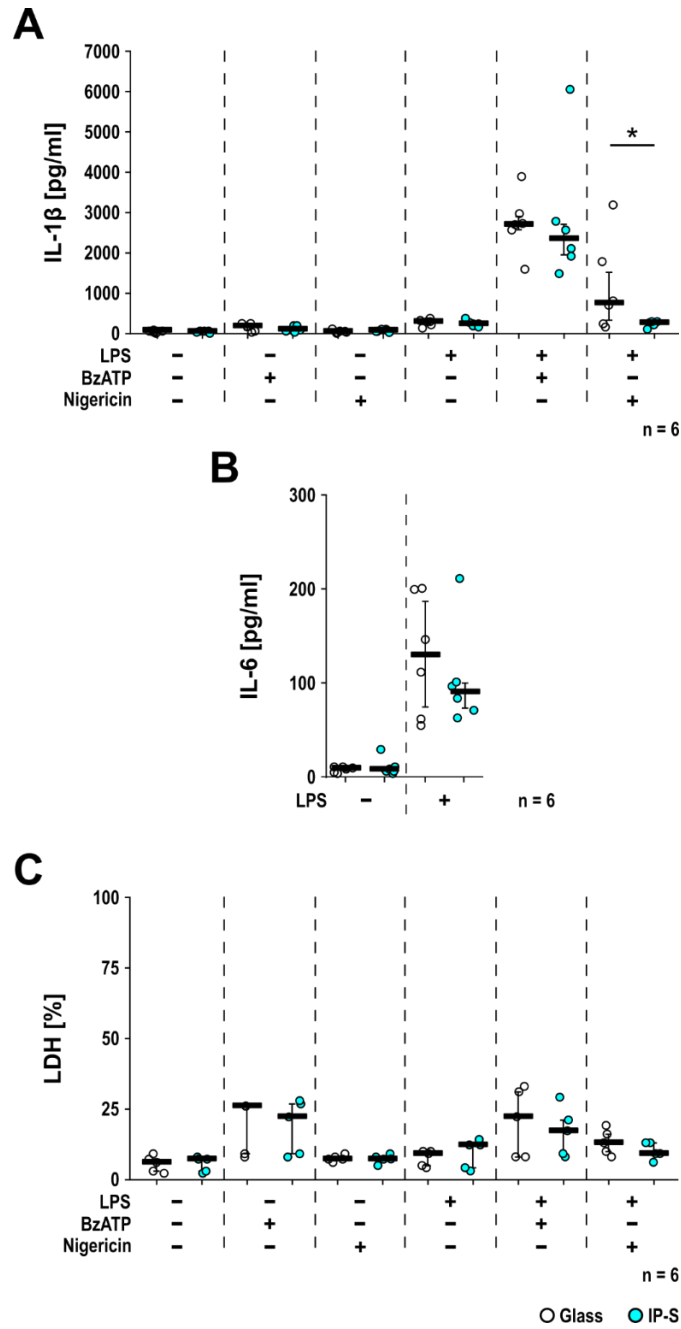


Figure 8: Cytokine concentrations and lactate dehydrogenase (LDH) activity in supernatants of THP-1 cell-derived M1-like macrophages on IP-S-printed surfaces, compared to glass. Cells were left either untreated or stimulated with 1 μ g/ml lipopolysaccharide (LPS) for 5 hours. After priming, 200 μ M 2'(3')-O-(4-benzoylbenzoyl)adenosine-5'-triphosphate (BzATP) or 50 μ M nigericin were applied for 40 minutes. (A) IL-1 β concentration, (B) IL-6 concentration, (C) LDH activity. Each dot represents an individual value and bars show the median. Whiskers correspond to the 25th and 75th percentiles, n = 5-6, * $p \leq 0.05$. Statistical analysis was performed using the Friedman test, followed by Wilcoxon signed-rank test for pairwise comparisons.

3.2 Characterization of macrophages to be used in co-culture for tube formation assay

3.2.1 Impact of culture time on the surface marker expression of macrophages

Macrophages were differentiated into 3 different subtypes: M0-, M1-, and M2-like macrophages. After their differentiation, they were either investigated immediately (0 hours of cultivation) or cultivated for 20 hours in R.

Before analyzing marker expression by flow cytometry, a gate was applied to sort out debris, dead cells and doublets from living and single cells. The debris and dead cells were excluded based on FSC-A (particle size) and SSC-A (granularity) parameters (Figure 9A). Then the doublets were eliminated by a new gate based on FSC-H and FSC-A first, and then SSC-H and SSC-A (Figure 9B,C). Therefore, only the events that were still inside of both gates (living single cells) were kept for further analysis (Figure 9D,E,F).

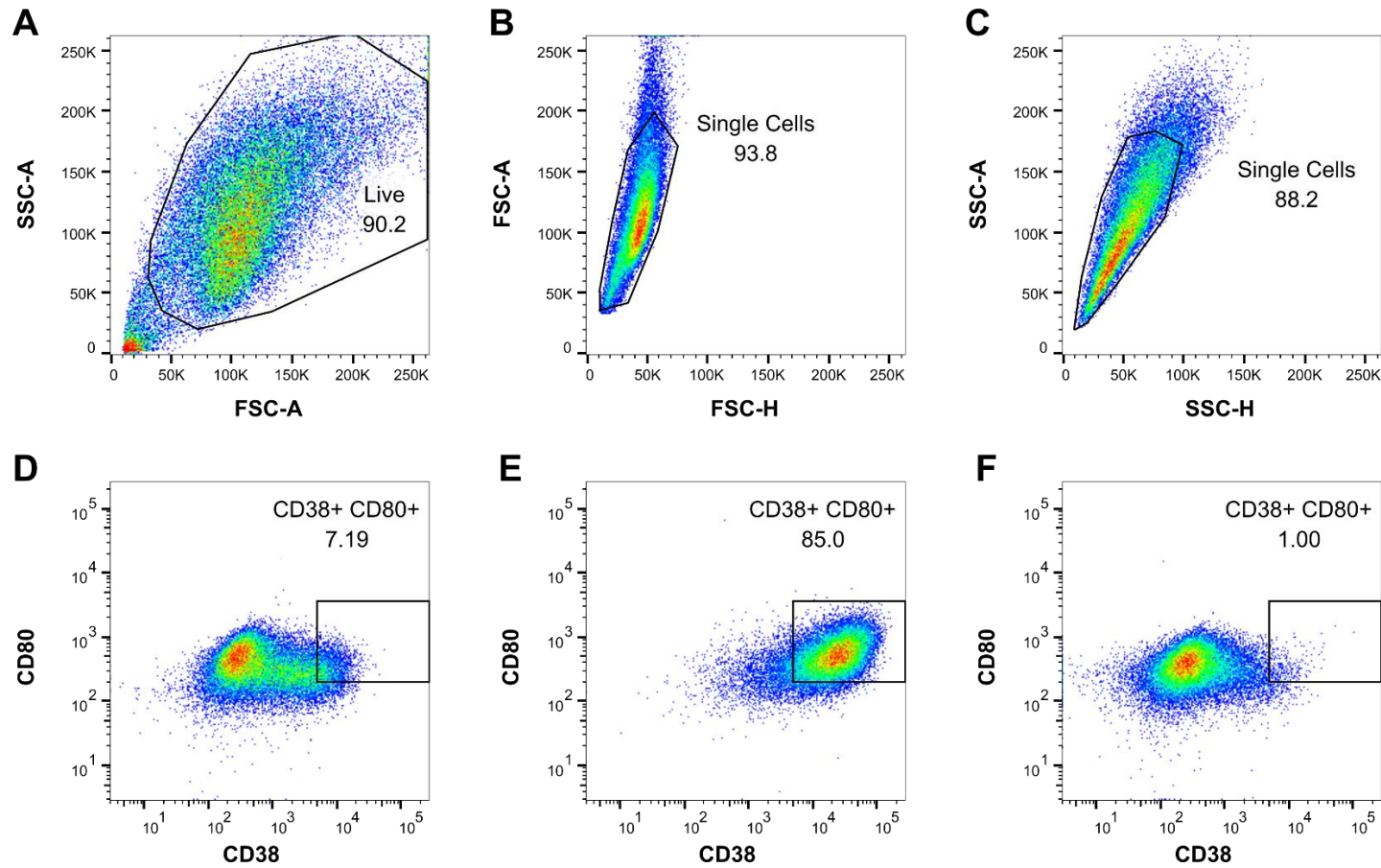


Figure 9: Example of the gates applied for debris and doublet discrimination and analysis of two cell surface markers. Cells (M0-like macrophages) were gated to eliminate debris and doublets with side-scatter-area (SSC-A) and forward-scatter (FSC)-A (A), FSC-A and FSC-height (H) (B), SSC-A and SSC-H (C). The gated M0- (D), M1-(E), and M2-like macrophages (F) were then analyzed for cell surface marker expression. Cluster of differentiation (CD)38 and CD80 are shown as an example. One passage out of six is used and cells were cultured for 6 hours.

Eight surface markers were measured by flow cytometry in order to determine whether macrophages underwent phenotypic changes after 20 hours of culture in comparison to 0 hours. Results for single markers are given in mean fluorescence intensity (MFI). When combinations of two markers were investigated, the percentage of double-positive cells among the total population is given.

CD80, CD86, CD38, and CD83 are typical M1 markers, CD209, CD163, and CD206 are M2 markers, while HLA-DR can be expressed by both subtypes (Wolf et al., 2025). In our experiments, CD83 was poorly expressed by all macrophage subtypes (Figure 10; 18-54.2 MFI). CD80 and CD38 were indeed over-expressed by the M1-like macrophages, but only CD38 showed a subtle but significant change in expression ($p = 0.046$). After 20 hours of culture, the MFI of CD38 was slightly reduced. CD86 and CD163 were expressed equally among all subtypes and did not display any changes over time (CD86, 431-11228 MFI; CD163, 76.7-768 MFI). HLA-DR was as expressed by M1-like and M2-like macrophages (M1-like, 723-1116 MFI; M2-like, 49.9-1344 MFI after 0 h of culture). It increased on the M2-like macrophages after 20 hours of culture (814-1521 MFI, $p = 0.028$). Similar changes were seen for CD206 (from 20-680 to 312-1155 MFI, $p = 0.028$). Although CD209 was more strongly expressed by the M2-like macrophages, the M1-like macrophages increased their expression after 20 hours of culture from 398-512 to 426-729 MFI ($p = 0.028$).

Most of the M1-like macrophages were CD38⁺/CD80⁺, CD38⁺/CD209⁻, CD206⁻/CD38⁺, CD206⁻CD38⁺, CD206⁻/HLA-DR⁻, which fits with the expected marker expression (Figure 11). M2-like macrophages showed more changes over time. They predominantly displayed the following phenotype: CD38⁻/CD209⁺, CD86⁺CD209⁺, CD206⁺/CD38⁻, and CD206⁺/HLA-DR⁺. The percentage of CD206⁺/CD38⁻ M2 macrophages increased after 20 hours of culture (from 4.07-18.1 to 18.3-28.1%, $p = 0.028$). Similar changes were seen for CD206⁺/HLA-DR⁺ macrophages (from 3.44-19.6 to 13.6-25.1%, $p = 0.046$).

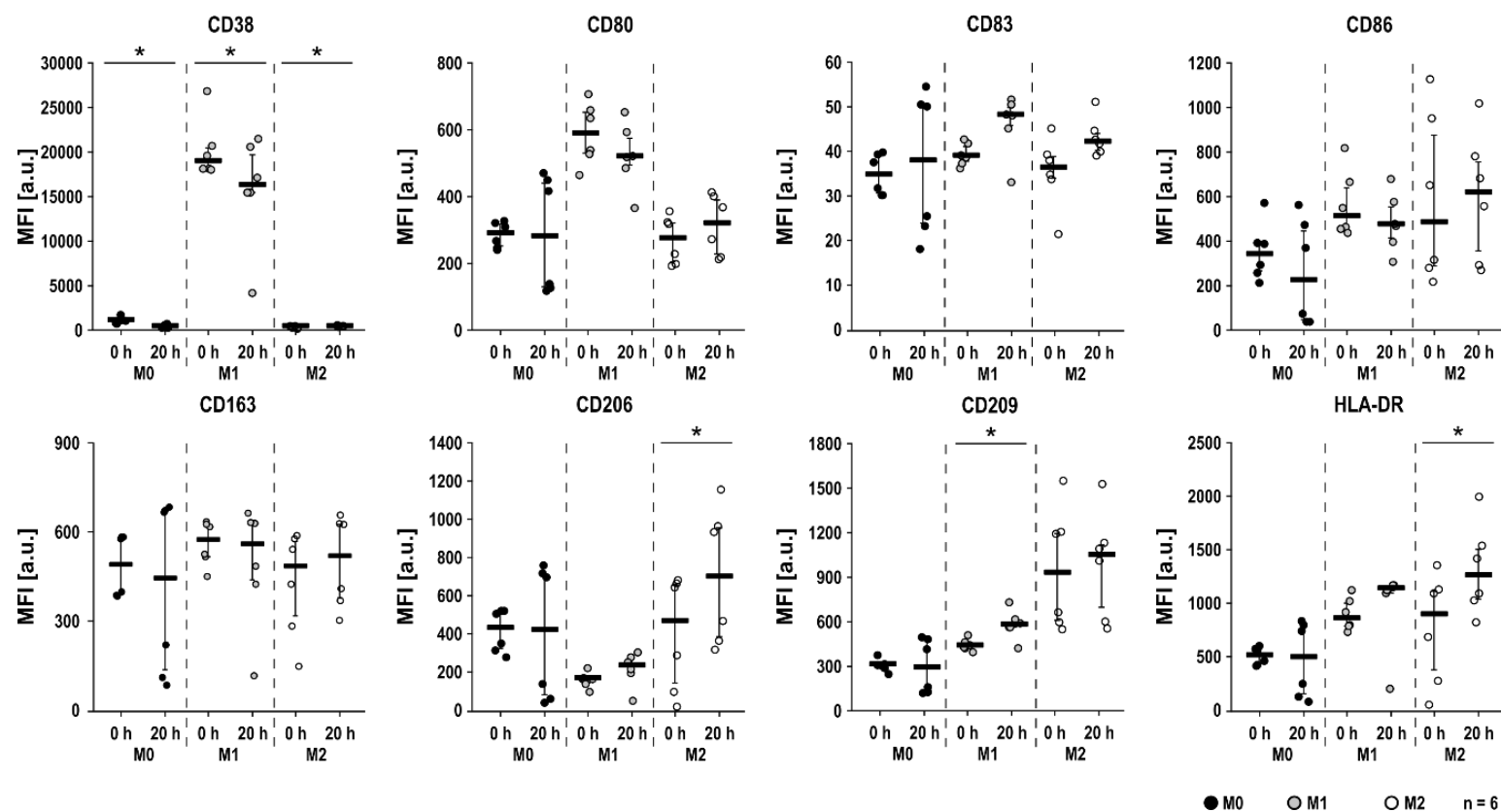


Figure 10: Mean fluorescence intensity (MFI) of M0-, M1- and M2-like macrophage surface markers. CD38, CD80, CD83, CD86, CD163, CD206, CD209 and HLA-DR were quantified on the three macrophages phenotypes immediately (0 h) or 20 hours (20 h) after their differentiation. a.u., arbitrary units; CD, cluster of differentiation; h, hour; HLA, human leukocyte antigen. Each dot represents an individual value and bars show the median. Whiskers correspond to the 25th and 75th percentiles, n = 6, * $p \leq 0.05$. Statistical analysis was performed using the Wilcoxon signed-rank test for pairwise comparisons.

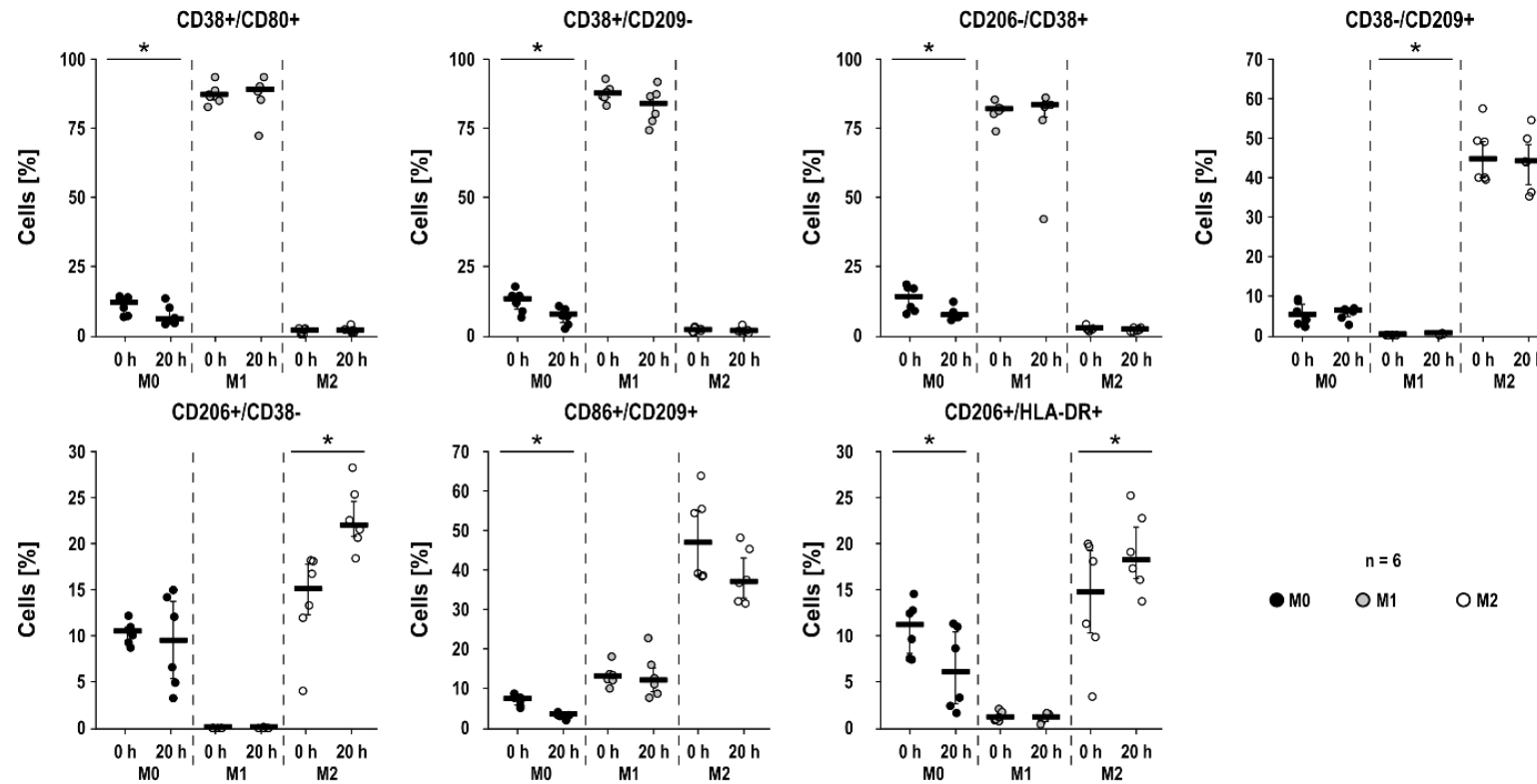


Figure 11: Proportion of M0-, M1-, and M2-like macrophages expressing combined surface markers. CD38, CD80, CD86, CD206, CD209 and HLA-DR were measured and combined. Y-axis displays the percentage of the population expressing the corresponding markers after 0 or 20 hours of cultivation following their differentiation. CD, cluster of differentiation; h, hour; HLA, human leukocyte antigen. Each dot represents an individual value and bars show the median. Whiskers correspond to the 25th and 75th percentiles, $n = 6$, * $p \leq 0.05$. Statistical analysis was performed using the Wilcoxon signed-rank test for pairwise comparisons.

There were slight but no systematic changes over time in the marker expression of M0-like macrophages (Figure 10, Figure 11).

3.2.2 M1-like macrophages in bE with cell detachment

The functions of polarized macrophages after cultivation over time and in different media were assessed by analyzing IL-1 β and IL-6 release. 50,000 THP-1 cell-derived M1-like macrophages were cultured in 96-well plates for 6 hours according to the original protocol for IL-1 β and IL-6 release experiments (Figure 2A) or for 20 hours to simulate co-culture experiments with HUVECs (Figure 2B). During this time, macrophages were kept in three different media: R (RPMI-1640 + 10% FBS), bE (ECGM-2 without growth factors), and E (ECGM-2 + 2% FBS + supplements). During this experiment, cells were detached with TrypLE after their differentiation from the flasks before being added into the culture wells for the stimulation of cytokine release as described in chapters 2.2.4 and 2.2.5.

After 6 hours of culture, IL-1 β concentrations remained below 22 pg/ml, when M1-like macrophages were not simulated at all, or below 286 pg/ml when they only received LPS, BzATP or nigericin (Figure 12A). In R, IL-1 β concentrations increased when macrophages were treated with BzATP or nigericin in addition to LPS. Under the same conditions, the IL-1 β concentrations were significantly lower after cultivating macrophages in bE and even more in E. P-values below 0.05 and corresponding IL-1 β concentrations after dual stimulation (LPS + BzATP or LPS + nigericin) are summarized in Table 2. Data are visualized in Figure 12A.

Table 2: Select IL-1 β concentrations after 6 hours of culture of M1-like macrophages in different media.

Condition		IL-1 β [pg/ml]	p (R vs bE)	p (R vs E)	p (bE vs E)
LPS + BzATP	R	377-1929	0.028	0.028	0.028
	bE	58-287			
	E	18-70			
LPS + nigericin	R	306-1590	0.028	0.028	0.046
	bE	227-950			
	E	71-366			

IL, interleukin; LPS, lipopolysaccharide; BzATP, 2'(3')-O-(4-benzoylbenzoyl)adenosine-5'-triphosphate; R, RPMI-1640 + 10% FBS; bE, basal ECGM-2 (ECGM-2 + 2% FBS); E, ECGM-2 + 2% FBS + supplements.

After 20 hours of culture, IL-1 β concentrations strongly decreased, in comparison to 6 hours culture. Values remained low when macrophages received no or only one stimulus (0-47 pg/ml) and reached 135 pg/ml in R, when cells were primed with LPS and activated with BzATP. The medium also influenced IL-1 β concentrations. When the cells were treated with LPS and BzATP, the concentration was highest in R, followed by bE and E. When cells were treated with LPS and nigericin, IL-1 β concentrations were higher in bE than in R or E, but the differences were only significant between R and bE and bE and E. P-values below 0.05 and corresponding IL-1 β concentrations after dual stimulation (LPS + BzATP or LPS + nigericin) are summarized in Table 3. Data are visualized in Figure 12B.

Table 3: Select IL-1 β concentrations after 20 hours of culture of M1-like macrophages in different media.

Condition		IL-1 β [pg/ml]	p (R vs bE)	p (R vs E)	p (bE vs E)
LPS + BzATP	R	11-135	0.048	0.028	0.026
	bE	8-30			
	E	5-23			
LPS + nigericin	R	31-79	0.027	0.345	0.046
	bE	61-132			
	E	20-124			

IL, interleukin; LPS, lipopolysaccharide; BzATP, 2'(3')-O-(4-benzoylbenzoyl)adenosine-5'-triphosphate; R, RPMI-1640 + 10% FBS; bE, basal (ECGM-2 + 2% FBS); E, ECGM-2 + 2% FBS + supplements.

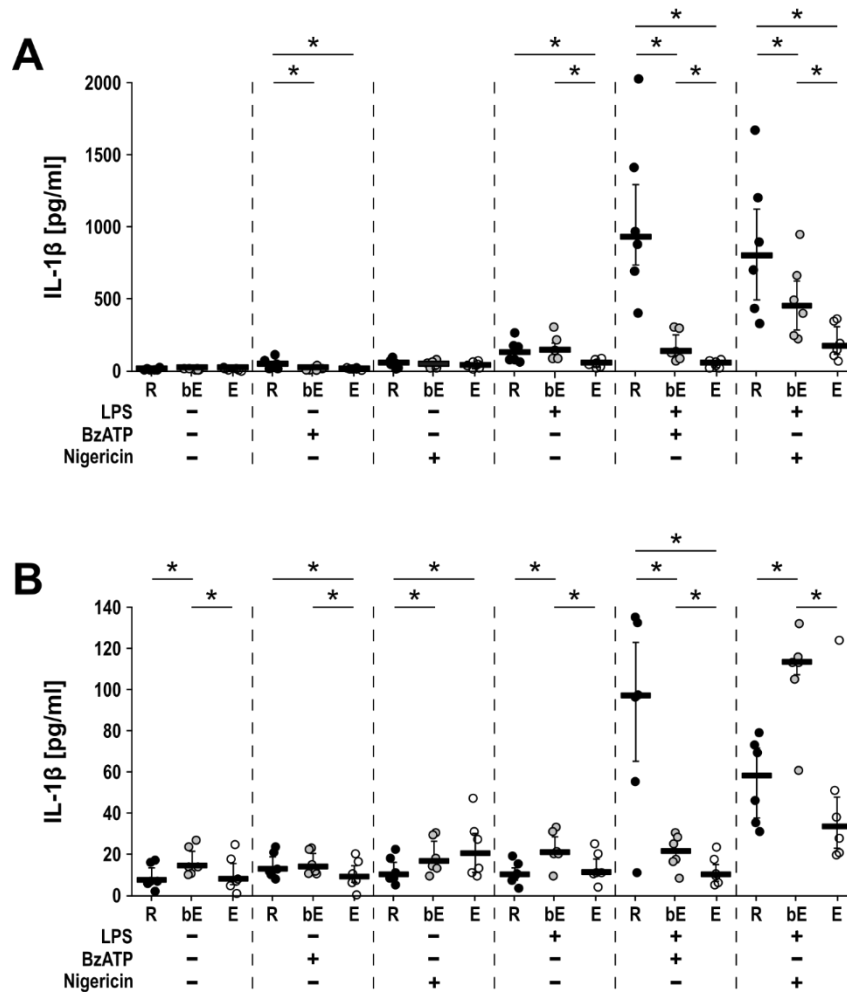


Figure 12: IL-1 β concentrations after 6 hours (A) and 20 hours (B) of culture of M1-like macrophages. Cells were left either untreated or stimulated with 1 μ g/ml lipopolysaccharide (LPS) for 5 hours. After priming, 200 μ M 2'(3')-O-(4-benzoylbenzoyl)adenosine-5'-triphosphate (BzATP) or 50 μ M nigericin were applied for 40 minutes. R, RPMI-1640 + 10% FBS; bE, basal ECGM-2 (ECGM-2 + 2% FBS); E, ECGM-2 + 2% FBS + supplements; each dot represents an individual value and bars show the median. Whiskers correspond to the 25th and 75th percentiles, n = 6, * $p \leq 0.05$. Statistical analysis was performed using the Friedman test, followed by Wilcoxon signed-rank test for pairwise comparisons.

In parallel to IL-1 β , IL-6 was measured after 6 and 20 hours of culture, in R, bE, and E. When cells were not treated with LPS, BzATP or nigericin, low IL-6 concentrations (0-26 pg/ml) were measured in cell culture supernatants. BzATP or nigericin alone did not induce high IL-6 concentrations either (0-36 pg/ml). However, the addition of LPS led to

increased concentrations in supernatants. IL-6 concentrations were similar in R and bE and did not depend on BzATP or nigericin addition. In bE, IL-6 concentrations were low and significantly lower than after culture in R or bE under the same conditions. P-values below 0.05 and corresponding IL-6 concentrations after LPS stimulation are summarized in Table 4. Data are visualized in Figure 13A.

Table 4: Select IL-6 concentrations after 6 hours of culture of M1-like macrophages in different media.

Condition		IL-6 [pg/ml]	p (R vs bE)	p (R vs E)	p (bE vs E)
LPS	R	83-373	0.344	0.028	0.028
	bE	98-411			
	E	0-34			
LPS + BzATP	R	83-414	0.463	0.028	0.028
	bE	81-325			
	E	0-37			
LPS + nigericin	R	67-477	0.753	0.028	0.028
	bE	88-347			
	E	8-57			

IL, interleukin; LPS, lipopolysaccharide; BzATP, 2'(3')-O-(4-benzoylbenzoyl)adenosine-5'-triphosphate; R, RPMI-1640 + 10% FBS; bE, basal ECGM-2 (ECGM-2 + 2% FBS); E, ECGM-2 + 2% FBS + supplements.

After 20 hours of culture, IL-6 concentrations did not exceed 99 pg/ml, irrespective of the experimental condition. Without LPS, IL-6 concentrations remained below 36 pg/ml. After stimulation with LPS, IL-6 concentrations were higher after culture in bE compared to R and E. Although no statistical testing was performed to avoid excessive multiple testing, the IL-6 release in response to LPS seemed to be considerably lower after 20 hours of culture compared to 6 hours (Figure 13). P-values below 0.05 and corresponding IL-6 concentrations after stimulation with LPS are summarized in Table 5. Data are visualized in Figure 13B.

Table 5: Select IL-6 concentrations after 20 hours of culture of M1-like macrophages.

Condition		IL-6 [pg/ml]	p (R vs bE)	p (R vs E)	p (bE vs E)
LPS	R	20-50	0.028	0.753	0.046
	bE	49-94			
	E	0-99			
LPS + BzATP	R	7-46	0.028	0.753	0.046
	bE	42-84			
	E	0-96			
LPS + nigericin	R	0-29	0.027	0.917	0.046
	bE	24-81			
	E	0-91			

IL, interleukin; LPS, lipopolysaccharide; BzATP, 2'(3')-O-(4-benzoylbenzoyl)adenosine-5'-triphosphate; R, RPMI-1640 + 10% FBS; bE, basal ECGM-2 (ECGM-2 + 2% FBS); E, ECGM-2 + 2% FBS + supplements.

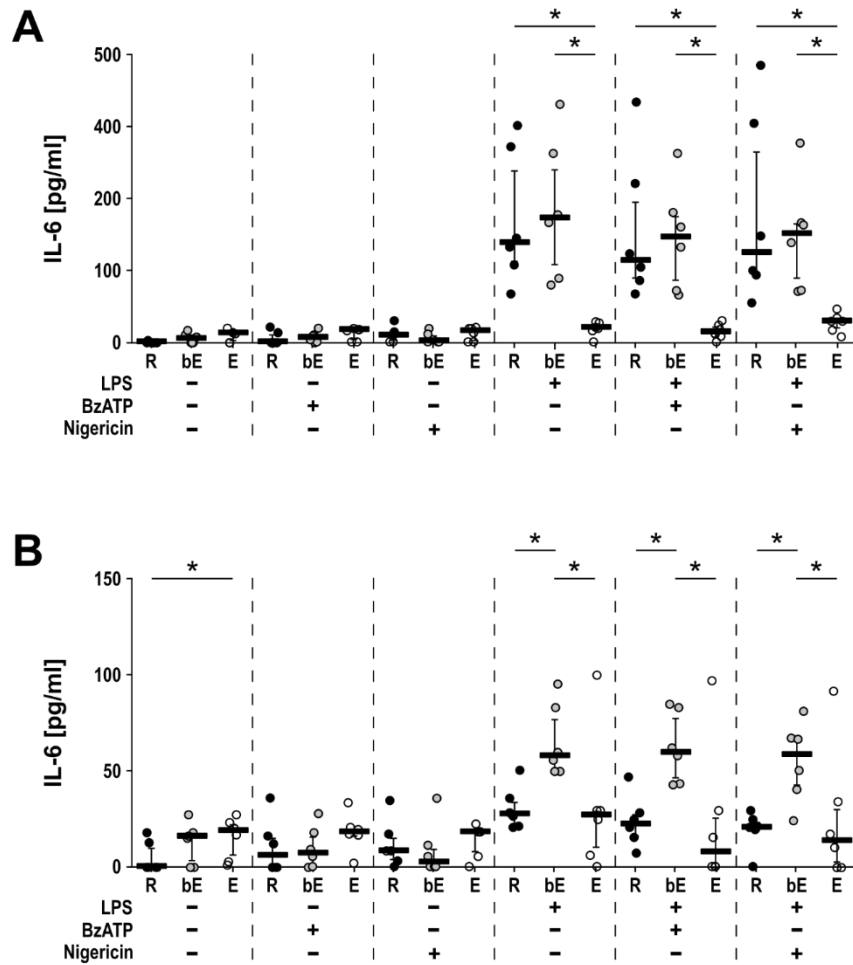


Figure 13: IL-6 concentration after 6 hours (A) and 20 hours (B) of culture of M1-like macrophages. Cells were left either untreated or stimulated with 1 $\mu\text{g/ml}$ lipopolysaccharide (LPS) for 5 hours. After priming, 200 μM 2'(3')-O-(4-benzoylbenzoyl)adenosine-5'-triphosphate (BzATP) or 50 μM nigericin were applied for 40 minutes. R, RPMI-1640 + 10% FBS; bE, basal ECGM-2 (ECGM-2 + 2% FBS); E, ECGM-2 + 2% FBS + supplements; each dot represents an individual value and bars show the median. Whiskers correspond to the 25th and 75th percentiles, $n = 6$, $* p \leq 0.05$. Statistical analysis was performed using the Friedman test, followed by Wilcoxon signed-rank test for pairwise comparisons.

Cell death after 6 hours of culture was lower in the absence of any stimuli (10-26%). It was higher in R than in bE and E when cells were treated with BzATP, with or without LPS. The media did not induce a different cell viability among the other conditions. P values below 0.05 as well as corresponding LDH activity are given in Table 6. Data are visualized in Figure 14A.

Table 6: Select lactate dehydrogenase (LDH activities) after 6 hours of culture of M1-like macrophages.

Condition		LDH (%)	p (R vs bE)	p (R vs E)	p (bE vs E)
BzATP	R	24-56	0.028	0.028	0.528
	bE	1-12			
	E	0-28			
LPS + BzATP	R	31-85	0.028	0.028	0.116
	bE	19-33			
	E	9-41			

IL, interleukin; LPS, lipopolysaccharide; BzATP, 2'(3')-O-(4-benzoylbenzoyl)adenosine-5'-triphosphate; R, RPMI-1640 + 10% FBS; bE, basal ECGM-2 (ECGM-2 + 2% FBS); E, ECGM-2 + 2% FBS + supplements.

After 20 hours of culture, overall cell death increased and showed a broader variability, ranging from 2 % to 100 % (Figure 14B).

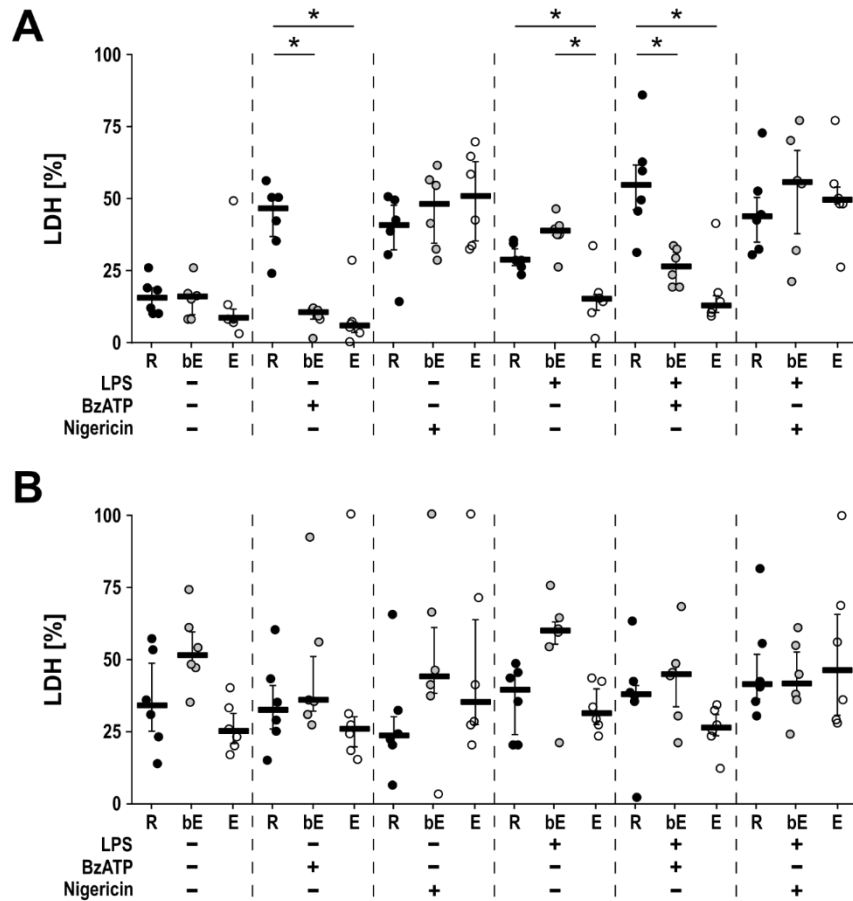


Figure 14: Lactate dehydrogenase (LDH) activity after 6 hours (A) and 20 hours (B) of culture of M1-like macrophages. Cells were left either untreated or stimulated with 1 $\mu\text{g/ml}$ lipopolysaccharide (LPS) for 5 hours. After priming, 200 μM 2'(3')-O-(4-benzoylbenzoyl)adenosine-5'-triphosphate (BzATP) or 50 μM nigericin were applied for 40 minutes. R, RPMI-1640 + 10% FBS; bE, basal ECGM-2 (ECGM-2 + 2% FBS); E, ECGM-2 + 2% FBS + supplements; each dot represents an individual value and bars show the median. Whiskers correspond to the 25th and 75th percentiles, $n = 6$, * $p \leq 0.05$. Statistical analysis was performed using the Friedman test, followed by Wilcoxon signed-rank test for pairwise comparisons.

3.2.3 M2-like macrophages in bE with cell detachment

IL-1 β and IL-6 release experiments as well as measurements of LDH activity were carried out in the same way as above, using M2-like macrophages instead of M1-like macrophages. After 6 hours of culture, IL-1 β did not exceed 162 pg/ml. Upon treatment with LPS and BzATP, the medium showed an effect akin to that observed in the previous

experiment with M1-like macrophages. IL-1 β concentrations were higher in R but low in bE and even lower E. Treating the cells with LPS and nigericin, did not have the same effect as IL-1 β was higher in bE than in R. P-values below 0.05 and corresponding IL-1 β concentrations after dual stimulation (LPS + BzATP or LPS + nigericin) are summarized in Table 7. Data are visualized in Figure 15A.

Table 7: Select IL-1 β concentrations after 6 hours of culture of M2-like macrophages in different media.

Condition		IL-1 β [pg/ml]	p (R vs bE)	p (R vs E)	p (bE vs E)
LPS + BzATP	R	58-90	0.027	0.028	0.026
	bE	6-11			
	E	2-6			
LPS + nigericin	R	37-177	0.027	0.028	0.028
	bE	66-162			
	E	17-46			

IL, interleukin; LPS, lipopolysaccharide; BzATP, 2'(3')-O-(4-benzoylbenzoyl)adenosine-5'-triphosphate); R, RPMI-1640 + 10% FBS; bE, basal ECGM-2 (ECGM-2 + 2% FBS); E, ECGM-2 + 2% FBS + supplements.

After 20 hours of culture, the cytokine concentrations showed a similar profile but were also higher. When the cells were treated with LPS and BzATP, IL-1 β was higher in R than in bE and E. When the cells were treated with LPS and nigericin, IL-1 β concentrations were higher in bE than in R or E. P-values below 0.05 and corresponding IL-1 β concentrations after dual stimulation (LPS + BzATP or LPS + nigericin) are summarized in Table 8. Data are visualized in Figure 15B.

Table 8: Select in IL-1 β concentrations after 6 hours of culture of M2-like macrophages in different media.

Condition		IL-1 β [pg/ml]	p (R vs bE)	p (R vs E)	p (bE vs E)
LPS + BzATP	R	122-363	0.043	0.043	0.176
	bE	4-18			
	E	1-10			
LPS + nigericin	R	53-234	0.043	0.138	0.043
	bE	171-620			
	E	33-261			

IL, interleukin; LPS, lipopolysaccharide; BzATP, 2'(3')-O-(4-benzoylbenzoyl)adenosine-5'-triphosphate; R, RPMI-1640 + 10% FBS; bE, basal ECGM-2 (ECGM-2 + 2% FBS); E, ECGM-2 + 2% FBS + supplements.

corresponding IL-6 concentrations under LPS stimulation are summarized in Table 9. Data are visualized in Figure 16A.

Table 9: Select IL-6 concentrations after 6 hours of culture of M2-like macrophages in different media.

Condition		IL-6 [pg/ml]	p (R vs bE)	p (R vs E)	p (bE vs E)
LPS	R	11-56	0.02	0.028	0.02
	bE	17-66			
	E	4-16			
LPS + BzATP	R	9-41	0.07	0.028	0.028
	bE	7-81			
	E	2-14			
LPS + nigericin	R	10-41	0.04	0.141	0.028
	bE	11-63			
	E	3-24			

IL, interleukin; LPS, lipopolysaccharide; BzATP, 2'(3')-O-(4-benzoylbenzoyl)adenosine-5'-triphosphate; R, RPMI-1640 + 10% FBS; bE, basal ECGM-2 (ECGM-2 + 2% FBS); E, ECGM-2 + 2% FBS + supplements.

After 20 hours of culture, the overall IL-6 concentrations increased upon LPS treatment. This time the maximal concentrations reached 398 pg/ml. However, bE still appeared to be the medium showing the highest IL-6 concentration, while it remained significantly lower and similar in R and E. P-values below 0.05 and corresponding IL-6 concentrations under LPS stimulation are summarized in Table 10. Data are visualized in Figure 16B.

Table 10: Select IL-6 concentrations after 20 hours of culture of M2-like macrophages in different media.

Condition		IL-6 [pg/ml]	p (R vs bE)	p (R vs E)	p (bE vs E)
LPS	R	48-142	0.043	0.279	0.043
	bE	161-347			
	E	16-169			
LPS + BzATP	R	59-98	0.043	0.500	0.043
	bE	127-263			
	E	15-156			
LPS + nigericin	R	53-118	0.028	0.917	0.043
	bE	131-398			
	E	21-171			

IL, interleukin; LPS, lipopolysaccharide; BzATP, 2'(3')-O-(4-benzoylbenzoyl)adenosine-5'-triphosphate; R, RPMI-1640 + 10% FBS; bE, basal ECGM-2 (ECGM-2 + 2% FBS); E, ECGM-2 + 2% FBS + supplements.

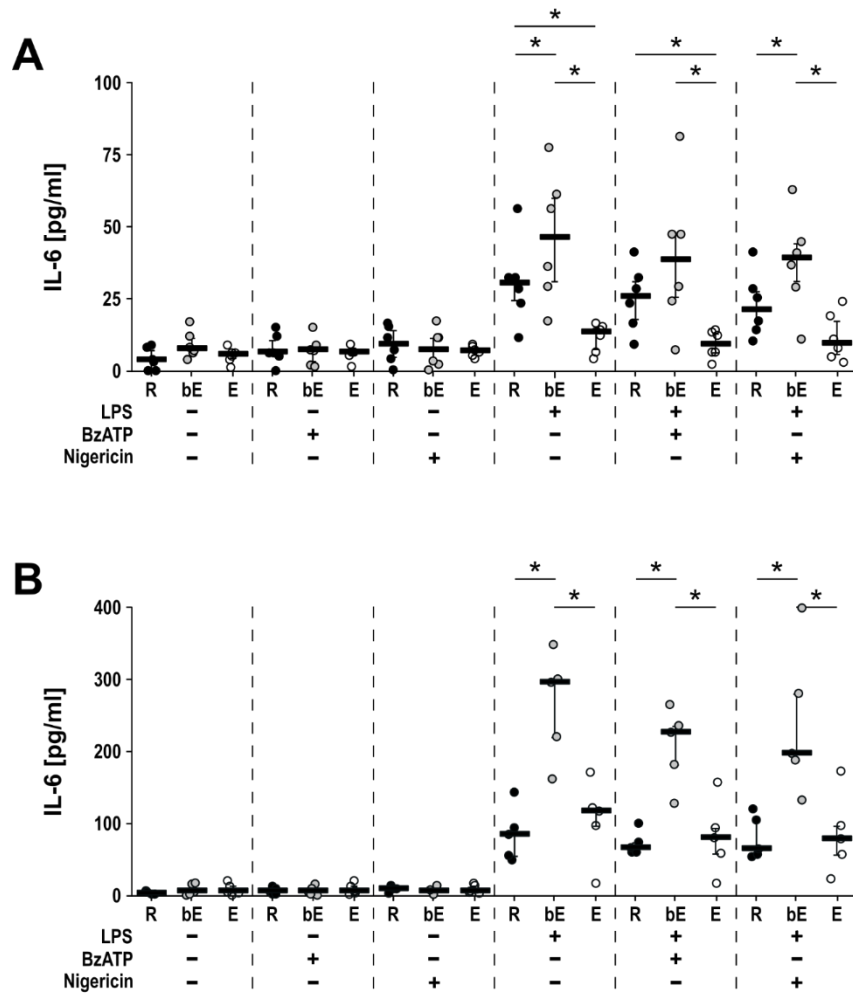


Figure 16: IL-6 concentration after 6 hours (A) and 20 hours (B) of culture of M2-like macrophages. Cells were left either untreated or stimulated with 1 $\mu\text{g/ml}$ lipopolysaccharide (LPS) for 5 hours. After priming, 200 μM 2'(3')-O-(4-benzoylbenzoyl)adenosine-5'-triphosphate (BzATP) or 50 μM nigericin were applied for 40 minutes. R, RPMI-1640 + 10% FBS; bE, basal ECGM-2 (ECGM-2 + 2% FBS); E, ECGM-2 + 2% FBS + supplements; each dot represents an individual value and bars show the median. Whiskers correspond to the 25th and 75th percentiles, $n = 5-6$, * $p \leq 0.05$. Statistical analysis was performed using the Friedman test, followed by Wilcoxon signed-rank test for pairwise comparisons.

LDH activity after 6 hours of culture was in about the same range like that after 20 hours (0-67%; Figure 17A, B). Some unsystematic statistically significant differences in LDH activity were seen.

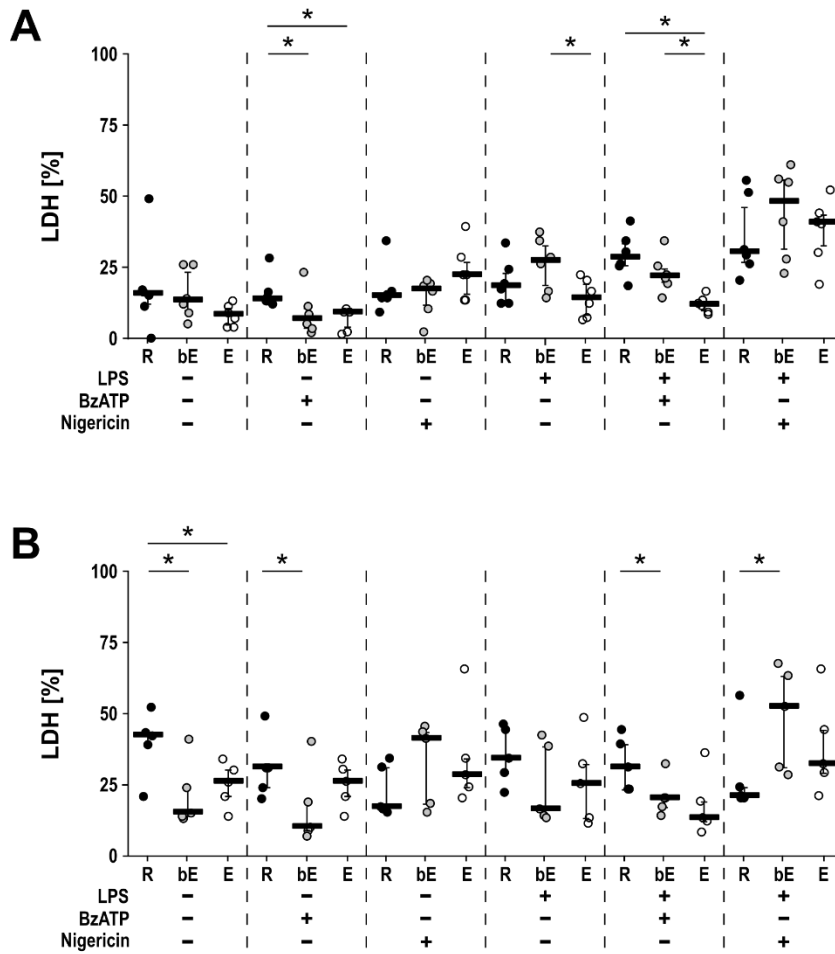


Figure 17: Lactate dehydrogenase (LDH) activity after 6 hours (A) and 20 hours (B) of culture of M2-like macrophages. Cells were left either untreated or stimulated with 1 $\mu\text{g}/\text{ml}$ lipopolysaccharide (LPS) for 5 hours. After priming, 200 μM 2'(3')-O-(4-benzoylbenzoyl)adenosine-5'-triphosphate (BzATP) or 50 μM nigericin were applied for 40 minutes. R, RPMI-1640 + 10% FBS; bE, basal ECGM-2 (ECGM-2 + 2% FBS); E, ECGM-2 + 2% FBS + supplements; each dot represents an individual value and bars show the median. Whiskers correspond to the 25th and 75th percentiles, $n = 5-6$, * $p \leq 0.05$. Statistical analysis was performed using the Friedman test, followed by Wilcoxon signed-rank test for pairwise comparisons

3.2.4 M1-like macrophages in bE without cell detachment

Before seeding cells into culture well plates, cells were detached from the surface of the flasks in which they were differentiated and polarized using TrypLE. This detachment process might influence the ability of macrophages to release cytokines. Therefore, the

same experiments as those described above were repeated but 0.1×10^6 cells were directly differentiated in 96-well plates followed by cytokine release experiments in the same well.

First, IL-1 β was measured after 6 hours in culture of M1-like macrophages in R, bE, and E. The cytokine release profile was similar to the one described previously (chapter 3.2.2), except that the concentrations were much higher (30-3782 pg/ml). IL-1 β concentrations were very high in R but significantly reduced in other media when the cells received LPS and BzATP. However, IL-1 β concentrations in bE and E were not significantly different. When the cells were treated with LPS and nigericin, IL-1 β concentrations in R and bE were similar and lower only in E. P-values below 0.05 and corresponding IL-1 β concentrations after dual stimulation (LPS + BzATP or LPS + nigericin) are summarized in Table 11. Data are visualized in Figure 18A.

Table 11: Select IL-1 β concentrations after 6 hours of culture of M1-like macrophages in different media.

Condition		IL-1 β [pg/ml]	p (R vs bE)	p (R vs E)	p (bE vs E)
LPS + BzATP	R	1520-3782	0.028	0.028	0.345
	bE	192-286			
	E	62-1912			
LPS + nigericin	R	1231-2461	0.075	0.028	0.028
	bE	1144-2142			
	E	435-1570			

IL, interleukin; LPS, lipopolysaccharide; BzATP, 2'(3')-O-(4-benzoylbenzoyl)adenosine-5'-triphosphate; R, RPMI-1640 + 10% FBS; bE, basal ECGM-2 (ECGM-2 + 2% FBS); E, ECGM-2 + 2% FBS + supplements.

After 20 hours of culture, IL-1 β concentrations decreased drastically in comparison to the 6 hours culture, as seen before (Figure 18B). However, the maximal release was again higher (14-901 pg/ml) than when the cells were detached (chapter 3.2.2). When the cells were treated with LPS and BzATP, IL-1 β concentrations were higher in R than in bE, or E. When the cells were treated with LPS and nigericin, IL-1 β concentrations were higher

in bE, while they were similar in R and E. P-values below 0.05 and corresponding IL-1 β concentrations after dual stimulation (LPS + BzATP or LPS + nigericin) are summarized in Table 12. Data are visualized in Figure 18B.

Table 12: Select IL-1 β concentrations after 20 hours of culture of M1-like macrophages in different media.

Condition		IL-1 β [pg/ml]	p (R vs bE)	p (R vs E)	p (bE vs E)
LPS + BzATP	R	529-901	0.028	0.028	0.027
	bE	44-75			
	E	23-46			
LPS + nigericin	R	277-383	0.028	0.027	0.028
	bE	320-541			
	E	54-122			

IL, interleukin; LPS, lipopolysaccharide; BzATP, 2'(3')-O-(4-benzoylbenzoyl)adenosine-5'-triphosphate; R, RPMI-1640 + 10% FBS; bE, basal ECGM-2 (ECGM-2 + 2% FBS); E, ECGM-2 + 2% FBS + supplements.

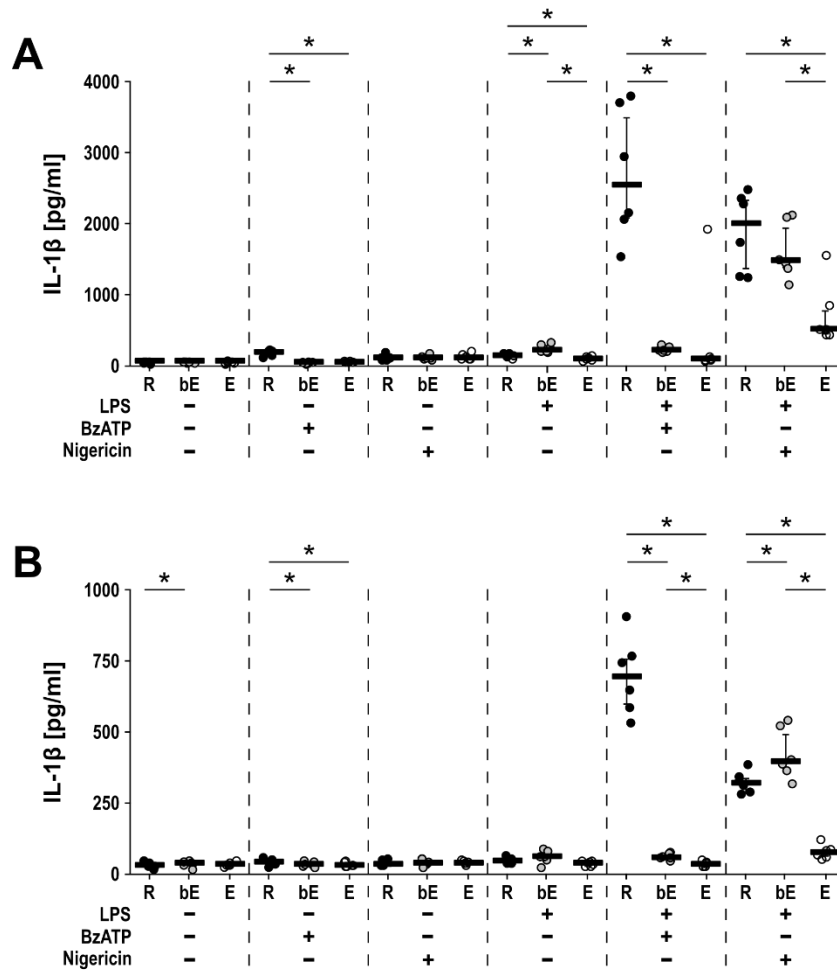


Figure 18: IL-1 β concentration after 6 hours (A) and 20 hours (B) of culture of M1-like macrophages. Cells were left either untreated or stimulated with 1 μ g/ml lipopolysaccharide (LPS) for 5 hours. After priming, 200 μ M 2'(3')-O-(4-benzoylbenzoyl)adenosine-5'-triphosphate (BzATP) or 50 μ M nigericin were applied for 40 minutes. R, RPMI-1640 + 10% FBS; bE, basal ECGM-2 (ECGM-2 + 2% FBS); E, ECGM-2 + 2% FBS + supplements; each dot represents an individual value and bars show the median. Whiskers correspond to the 25th and 75th percentiles, n = 6, * $p \leq 0.05$. Statistical analysis was performed using the Friedman test, followed by Wilcoxon signed-rank test for pairwise comparisons.

IL-6 concentrations were also 18 times higher (21-8844 pg/ml) than they were after 6 hours of culture (chapter 3.2.2). When M1-like macrophages were cultivated in R or bE, but also treated with LPS, with or without BzATP or nigericin, IL-6 concentrations were high. In E, or in the absence of LPS, IL-6 concentrations stayed very low. P-values below

0.05 and corresponding IL-6 concentrations under LPS stimulation are summarized in Table 13. Data are visualized in Figure 19A.

Table 13: Select IL-6 concentrations after 6 hours of culture of M1-like macrophages in different media.

Condition		IL-6 [pg/ml]	p (R vs bE)	p (R vs E)	p (bE vs E)
LPS	R	5994-8844	0.249	0.028	0.028
	bE	4954-7373			
	E	82-1103			
LPS + BzATP	R	3345-7409	0.249	0.028	0.028
	bE	4540-7148			
	E	133-1407			
LPS + nigericin	R	4121-7936	0.027	0.917	0.046
	bE	3504-7166			
	E	139-1450			

IL, interleukin; LPS, lipopolysaccharide; BzATP, 2'(3')-O-(4-benzoylbenzoyl)adenosine-5'-triphosphate; R, RPMI-1640 + 10% FBS; bE, basal ECGM-2 (ECGM-2 + 2% FBS); E, ECGM-2 + 2% FBS + supplements.

After 20 hours of culture, the IL-6 released dropped overall. Upon LPS treatment, most IL-6 was released in bE, while IL-6 concentrations in R and E were similar. They were however, higher than when the cells didn't receive LPS. P-values below 0.05 and corresponding IL-6 concentrations after LPS stimulation are summarized in Table 14. Data are visualized in Figure 19B.

Table 14: Select IL-6 concentrations after 20 hours of culture of M1-like macrophages in different media.

Condition		IL-6 [pg/ml]	p (R vs bE)	p (R vs E)	p (bE vs E)
LPS	R	27-452	0.116	0.046	0.046
	bE	14-1439			
	E	67-109			
LPS + BzATP	R	115-419	0.028	0.028	0.028
	bE	484-1501			
	E	37-77			
LPS + nigericin	R	183-485	0.028	0.028	0.028
	bE	505-1398			
	E	37-84			

IL, interleukin; LPS, lipopolysaccharide; BzATP, 2'(3')-O-(4-benzoylbenzoyl)adenosine-5'-triphosphate; R, RPMI-1640 + 10% FBS; bE, basal ECGM-2 (ECGM-2 + 2% FBS); E, ECGM-2 + 2% FBS + supplements.

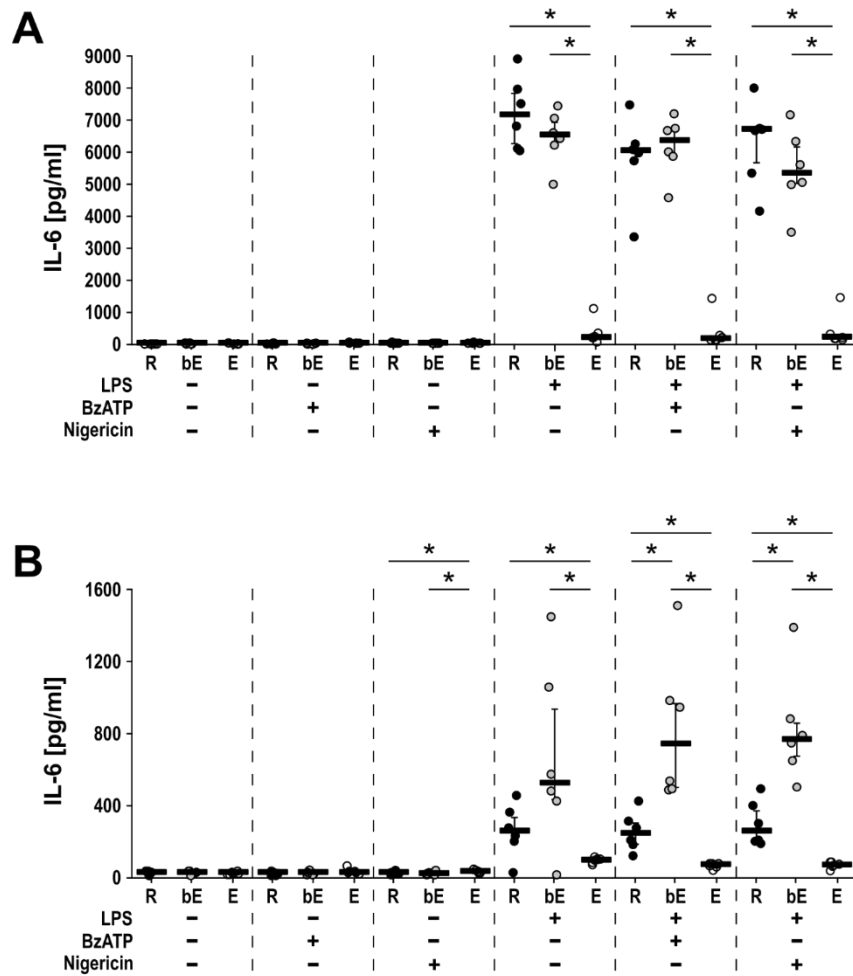


Figure 19: IL-6 concentration after 6 hours (A) and 20 hours (B) of culture of M1-like macrophages. Cells were left either untreated or stimulated with 1 μ g/ml lipopolysaccharide (LPS) for 5 hours. After priming, 200 μ M 2'(3')-O-(4-benzoylbenzoyl)adenosine-5'-triphosphate (BzATP) or 50 μ M nigericin were applied for 40 minutes. R, RPMI-1640 + 10% FBS; bE, basal ECGM-2 (ECGM-2 + 2% FBS); E, ECGM-2 + 2% FBS + supplements; each dot represents an individual value and bars show the median. Whiskers correspond to the 25th and 75th percentiles, n = 6, * $p \leq 0.05$. Statistical analysis was performed using the Friedman test, followed by Wilcoxon signed-rank test for pairwise comparisons.

LDH activity showed some statistically significant differences among the culture media and treatments (Figure 20A, B). In combination with BzATP, R resulted in higher LDH activity compared to media containing ECGM-2, irrespective of the duration of cultivation. When nigericin was present, higher LDH activity was seen in ECGM-2--containing media throughout.

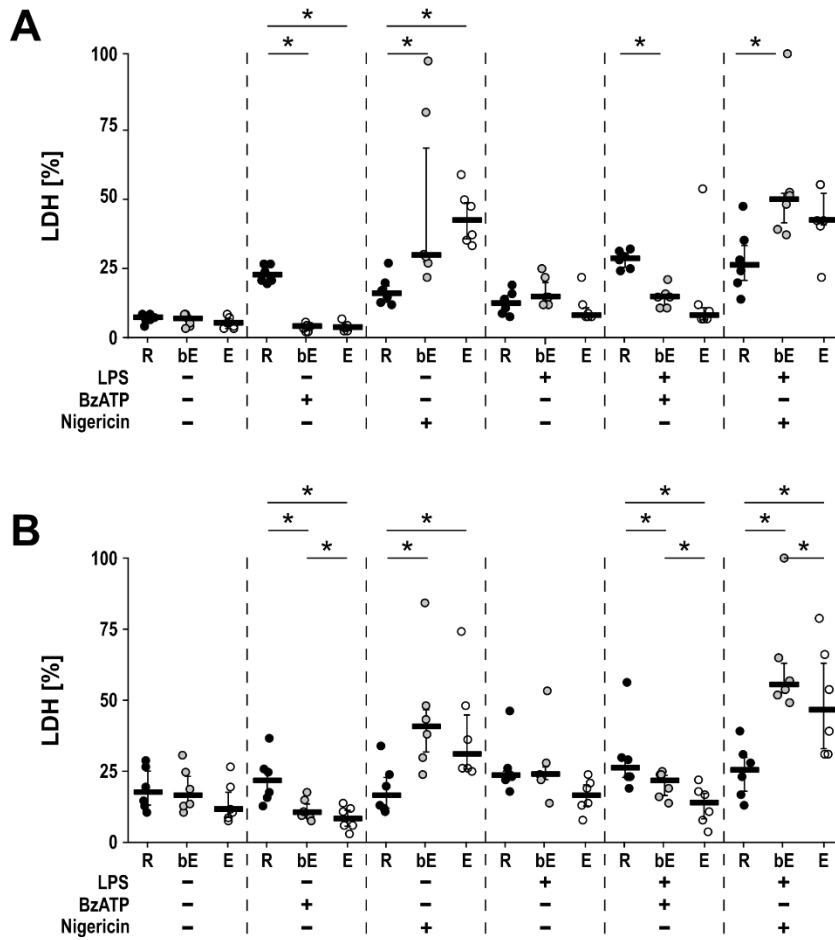


Figure 20: Lactate dehydrogenase (LDH) activity after 6 hours (A) and 20 hours (B) of culture of M1-like macrophages. Cells were left either untreated or stimulated with 1 $\mu\text{g/ml}$ lipopolysaccharide (LPS) for 5 hours. After priming, 200 μM 2'(3')-O-(4-benzoylbenzoyl)adenosine-5'-triphosphate (BzATP) or 50 μM nigericin were applied for 40 minutes. R, RPMI-1640 + 10% FBS; bE, basal ECGM-2 (ECGM-2 + 2% FBS); E, ECGM-2 + 2% FBS + supplements; each dot represents an individual value and bars show the median. Whiskers correspond to the 25th and 75th percentiles, $n = 6$, * $p \leq 0.05$. Statistical analysis was performed using the Friedman test, followed by Wilcoxon signed-rank test for pairwise comparisons.

3.2.5 M2-like macrophages in bE without cell detachment

Unlike M1-like macrophages, detachment did not raise IL-1 β concentration in supernatants of M2-like macrophages, with a maximum of 134 pg/mL after 6 hours of

culture. In this condition, IL-1 β concentrations were not significantly different between the culture media when the cells were treated with LPS and BzATP. However, when they were treated with LPS and nigericin, IL-1 β was higher in bE than in R and E. P-values below 0.05 and corresponding IL-1 β concentrations after dual stimulation (LPS + BzATP or LPS + nigericin) are summarized in Table 15. Data are visualized in Figure 21A.

Table 15: Select IL-1 β concentrations after 6 hours of culture of M2-like macrophages in different media.

Condition		IL-1 β [pg/ml]	p (R vs bE)	p (R vs E)	p (bE vs E)
LPS + BzATP	R	3-129	0.075	0.075	0.345
	bE	12-37			
	E	6-61			
LPS + nigericin	R	50-83	0.027	0.028	0.028
	bE	64-134			
	E	17-58			

IL, interleukin; LPS, lipopolysaccharide; BzATP, 2'(3')-O-(4-benzoylbenzoyl)adenosine-5'-triphosphate; R, RPMI-1640 + 10% FBS; bE, basal ECGM-2 (ECGM-2 + 2% FBS); E, ECGM-2 + 2% FBS + supplements.

After 20 hours of culture, IL-1 β concentrations were also different depending on the treatment. Overall concentrations increased in comparison to the 6 hours culture. When the cells were treated with LPS and BzATP, IL-1 β concentrations were higher in R than the other media, where the concentrations were similar to the ones without stimuli. After treatment with LPS and nigericin, highest IL-1 β concentrations were measured in bE, followed by R and E. P-values below 0.05 and corresponding IL-1 β concentrations after dual stimulation (LPS + BzATP or LPS + nigericin) are summarized in Table 16. Data are visualized in Figure 21B.

Table 16: Select IL-1 β concentrations after 6 hours of culture of M2-like macrophages in different media.

Condition		IL-1 β [pg/ml]	p (R vs bE)	p (R vs E)	p (bE vs E)
LPS + BzATP	R	154-342	0.028	0.028	0.026
	bE	19-26			
	E	8-11			
LPS + nigericin	R	86-146	0.028	0.027	0.028
	bE	157-286			
	E	24-82			

IL, interleukin; LPS, lipopolysaccharide; BzATP, 2'(3')-O-(4-benzoylbenzoyl)adenosine-5'-triphosphate; R, RPMI-1640 + 10% FBS; bE, basal ECGM-2 (ECGM-2 + 2% FBS); E, ECGM-2 + 2% FBS + supplements.

corresponding IL-6 concentrations under LPS stimulation are summarized in Table 17. Data are visualized in Figure 22A.

Table 17: Select IL-6 concentrations after 6 hours of culture of M2-like macrophages in different media.

Condition		IL-6 [pg/ml]	p (R vs bE)	p (R vs E)	p (bE vs E)
LPS	R	72-203	0.046	0.046	0.028
	bE	146-293			
	E	27-80			
LPS + BzATP	R	82-168	0.028	0.028	0.028
	bE	128-266			
	E	20-74			
LPS + nigericin	R	75-243	0.028	0.028	0.028
	bE	125-244			
	E	24-78			

IL, interleukin; LPS, lipopolysaccharide; BzATP, 2'(3')-O-(4-benzoylbenzoyl)adenosine-5'-triphosphate; R, RPMI-1640 + 10% FBS; bE, basal ECGM-2 (ECGM-2 + 2% FBS); E, ECGM-2 + 2% FBS + supplements.

After 20 hours of culture, the overall IL-6 concentration increased, as well as the difference between the concentrations in bE and the other media. bE was still the medium in which IL-6 concentrations were the highest, while IL-6 concentrations in R were either similar or slightly higher than in E. P-values below 0.05 and corresponding IL-6 concentrations under LPS stimulation are summarized in Table 18. Data are visualized in Figure 22B.

Table 18: Select IL-6 concentrations after 20 hours of culture of M2-like macrophages in different media.

Condition		IL-6 [pg/ml]	p (R vs bE)	p (R vs E)	p (bE vs E)
LPS	R	127-189	0.028	0.028	0.028
	bE	203-391			
	E	88-176			
LPS + BzATP	R	90-218	0.027	0.463	0.028
	bE	200-398			
	E	81-180			
LPS + nigericin	R	71-201	0.027	0.116	0.027
	bE	211-301			
	E	84-168			

IL, interleukin; LPS, lipopolysaccharide; BzATP, 2'(3')-O-(4-benzoylbenzoyl)adenosine-5'-triphosphate; R, RPMI-1640 + 10% FBS; bE, basal ECGM-2 (ECGM-2 + 2% FBS); E, ECGM-2 + 2% FBS + supplements.

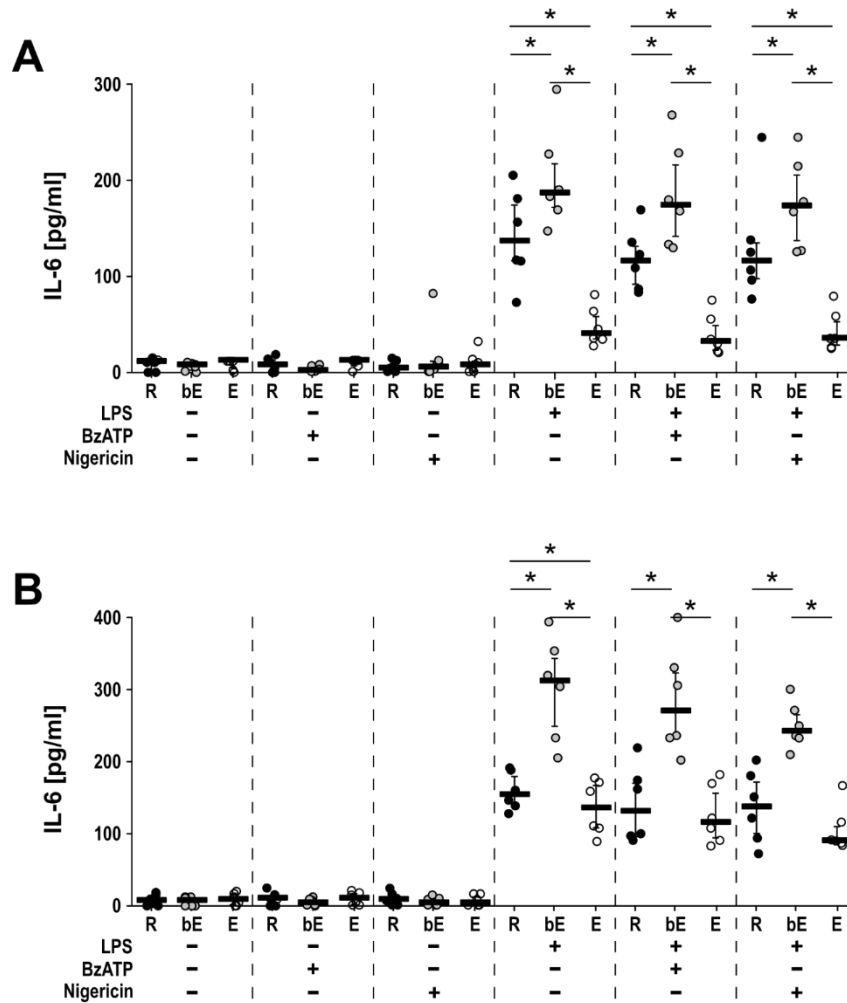


Figure 22: IL-6 concentration after 6 hours (A) and 20 hours (B) of culture of M2-like macrophages. Cells were left either untreated or stimulated with 1 µg/ml lipopolysaccharide (LPS) for 5 hours. After priming, 200 µM 2'(3')-O-(4-benzoylbenzoyl)adenosine-5'-triphosphate (BzATP) or 50 µM nigericin were applied for 40 minutes. R, RPMI-1640 + 10% FBS; bE, basal ECGM-2 (ECGM-2 + 2% FBS); E, ECGM-2 + 2% FBS + supplements; each dot represents an individual value and bars show the median. Whiskers correspond to the 25th and 75th percentiles, n = 6, * p ≤ 0.05. Statistical analysis was performed using the Friedman test, followed by Wilcoxon signed-rank test for pairwise comparisons.

LDH activity remained stable and low after 6 and 20 hours of culture, when FBS was present in the culture media. Only in bE upon treatment with LPS + nigericin, high levels of LDH activity were measured (49-81% after 6 hours, 39-100% after 20 hours), which significantly differed from media containing FBS after 6 hours of culture (Figure 23A,B).

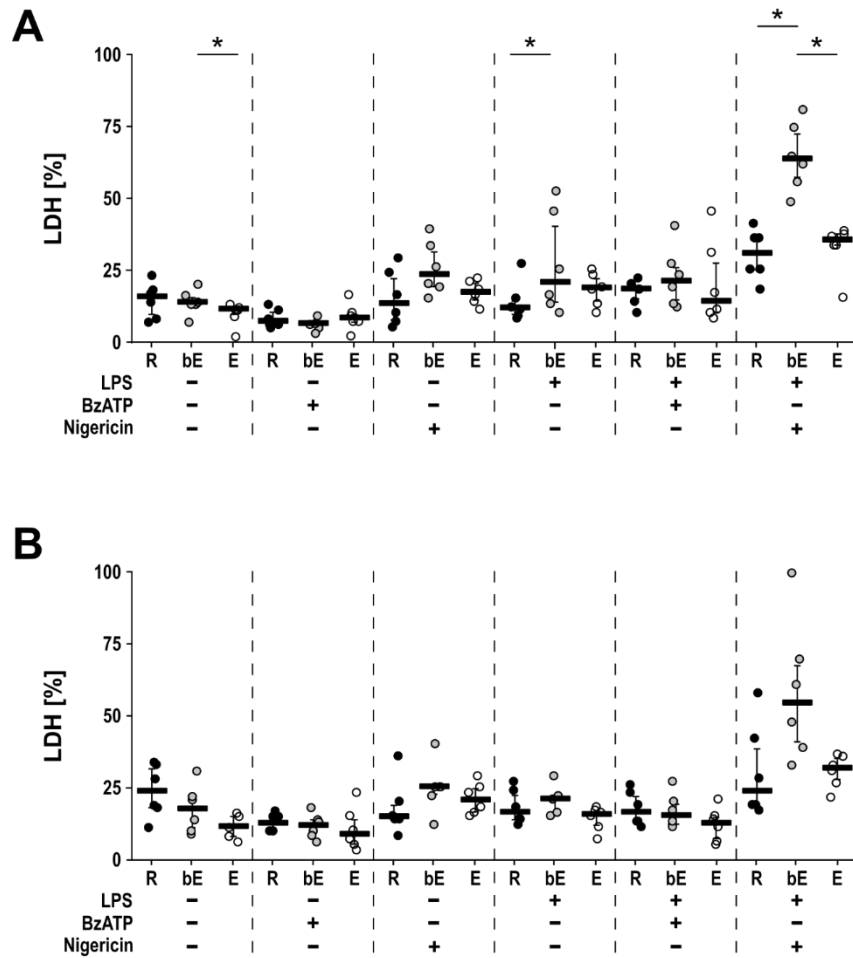


Figure 23: LDH activity after 6 hours (A) and 20 hours (B) of culture of M2-like macrophages. Cells were left either untreated or stimulated with 1 $\mu\text{g/ml}$ lipopolysaccharide (LPS) for 5 hours. After priming, 200 μM 2'(3')-O-(4-benzoylbenzoyl)adenosine-5'-triphosphate (BzATP) or 50 μM nigericin were applied for 40 minutes. R, RPMI-1640 + 10% FBS; bE, basal ECGM-2 (ECGM-2 + 2% FBS); E, ECGM-2 + 2% FBS + supplements; each dot represents an individual value and bars show the median. Whiskers correspond to the 25th and 75th percentiles, $n = 5-6$, * $p \leq 0.05$. Statistical analysis was performed using the Friedman test, followed by Wilcoxon signed-rank test for pairwise comparisons.

3.2.6 M1- and M2-like macrophages in a mix of R and E with cell detachment

After assessing in the previous chapter how bE impacts cytokine release and cell viability, this chapter focuses on the same endpoints in M1- and M2-like macrophages grown in a

1:2 mix medium of E and R. Cytokine release experiments were only performed after 20 hours of culture.

Starting with the M1-like macrophages, IL-1 β concentrations stayed low and stable among the different media with one or no stimulus. Upon LPS and BzATP treatment, IL-1 β showed the highest concentrations in R and low concentrations in the other two media, comparable to conditions in which no stimuli were applied. However, upon LPS and nigericin treatment, IL-1 β concentrations remained low in every media, including E+R. Upon stimulation with LPS, IL-6 concentrations were either similar in R and in E+R or lower in E+R. It was the lowest in E. There was no significant effect of the media on cell viability.

P-values below 0.05 and corresponding cytokine concentrations are presented in Table 19. Only data obtained under the required stimulation conditions for cytokine induction are shown. Data are visualized in Figure 24.

Table 19: Select cytokine concentrations after 20 hours of culture of M1-like macrophages in different media.

Condition		IL-1 β [pg/ml]	p (R vs bE)	p (R vs E)	p (E+R vs E)
LPS + BzATP	R	42-180	0.028	0.028	0.075
	E+R	27-106			
	E	23-65			
LPS + nigericin	R	20-59	0.027	0.292	0.116
	E+R	30-107			
	E	23-63			
Condition		IL-6 [pg/ml]	p (R vs bE)	p (R vs E)	p (E+R vs E)
LPS	R	29-127	0.028	0.046	0.116
	E+R	22-72			
	E	18-41			
LPS + BzATP	R	28-129	0.172	0.028	0.028
	E+R	16-70			
	E	6-41			
LPS + nigericin	R	39-120	0.116	0.028	0.028
	E+R	18-61			
	E	0-53			

IL, interleukin; LPS, lipopolysaccharide; BzATP, 2'(3')-O-(4-benzoylbenzoyl)adenosine-5'-triphosphate; R, RPMI-1640 + 10% FBS; E+R, 1:2 mix ECGM-2 + 2% FBS and RPMI-1640 + 10% FBS; E, ECGM-2 + 2% FBS + supplements.

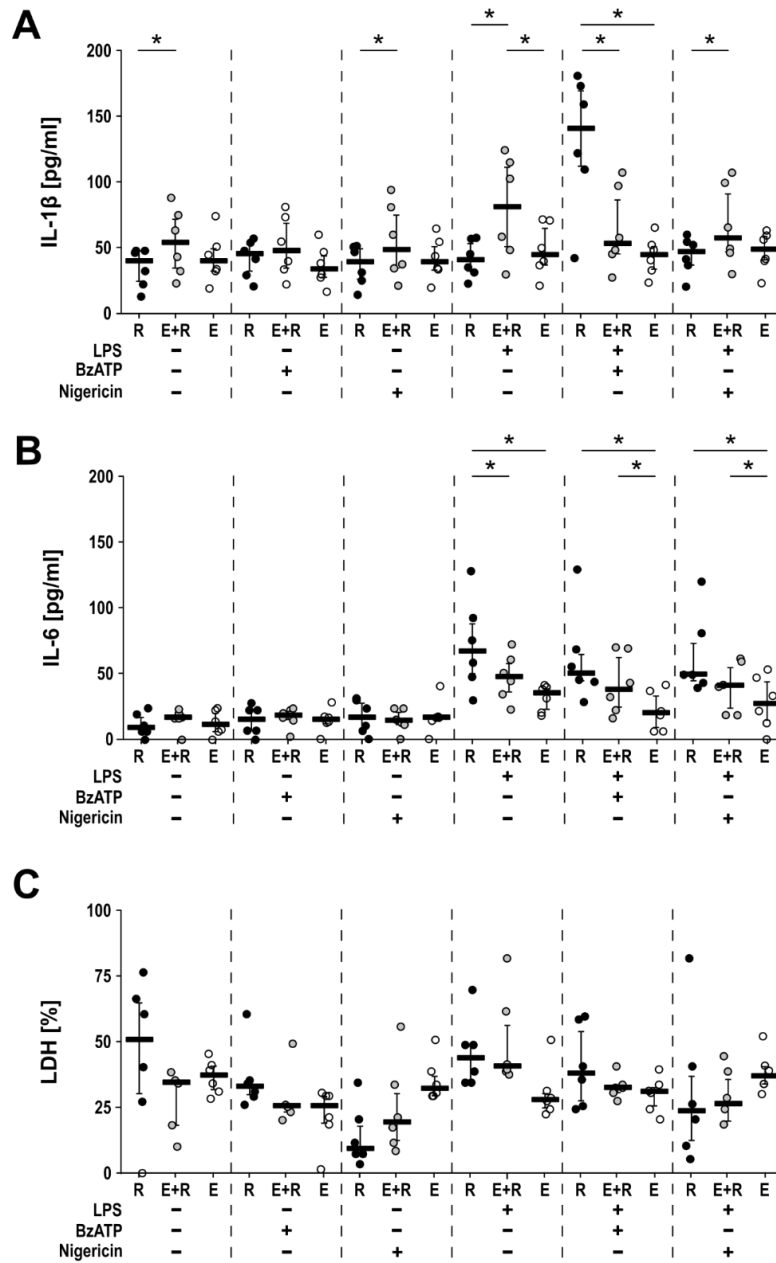


Figure 24: Cytokine concentration after 20 hours (B) of culture of M1-like macrophages. Cells were left either untreated or stimulated with 1 μ g/ml lipopolysaccharide (LPS) for 5 hours. After priming, 200 μ M 2'(3')-O-(4-benzoylbenzoyl)adenosine-5'-triphosphate (BzATP) or 50 μ M nigericin were applied for 40 minutes. (A) IL-1 β concentration, (B) IL-6 concentration, (C) LDH activity R, RPMI-1640 + 10% FBS; E+R, 1:2 mix RPMI-1640 + 10% FBS and ECGM-2 + 2% FBS + supplement; E, ECGM-2 + 2% FBS + supplements; each dot represents an individual value and bars show the median. Whiskers correspond to the 25th and 75th percentiles, n = 5-6, * $p \leq 0.05$. Statistical analysis was performed using the Friedman test, followed by Wilcoxon signed-rank test for pairwise comparisons.

IL-1 β stayed low for M2-like macrophages, except in R and upon treatment with LPS + BzATP. IL-1 β was between 6 and 672 pg/ml, which is higher than the concentration under the same condition for M1-like macrophages (Table 19). Just as for the M1-like macrophages, the IL-1 β concentrations were higher in R, than in E+R and lowest in E. IL-6 also showed higher concentrations in R than in the other media upon treatment with LPS (independent of BzATP or nigericin). However, it was intermediate in E+R and the lowest in E without being as low as without any LPS. LDH remained unchanged among the media.

LDH remained unchanged among the media.

P-values below 0.05 and corresponding IL-6 concentrations after stimulation with LPS are summarized in Table 20. Data are visualized in Figure 25.

Table 20: Select IL-6 concentrations after 20 hours of culture of M2-like macrophages in different media.

Condition		IL-1 β [pg/ml]	p (R vs bE)	p (R vs E)	p (E+R vs E)
LPS + BzATP	R	6-672	0.028	0.028	0.141
	E+R	3-69			
	E	2-7			
LPS + nigericin	R	4-59	0.674	0.207	0.293
	E+R	3-31			
	E	5-15			
Condition		IL-6 [pg/ml]	p (R vs bE)	p (R vs E)	p (E+R vs E)
LPS	R	130-353	0.917	0.046	0.116
	E+R	49-425			
	E	75-210			
LPS + BzATP	R	149-496	0.046	0.028	0.043
	E+R	101-410			
	E	64-173			
LPS + nigericin	R	167-487	0.028	0.028	0.028
	E+R	91-286			
	E	58-258			

IL, interleukin; LPS, lipopolysaccharide; BzATP, 2'(3')-O-(4-benzoylbenzoyl)adenosine-5'-triphosphate; R, RPMI-1640 + 10% FBS; E+R, 1:2 mix ECGM-2 + 2% FBS and RPMI-1640 + 10% FBS; E, ECGM-2 + 2% FBS + supplements.

3.2.7 M1- and M2-like macrophages in a mix of R and E without cell detachment

The same experiment was repeated without detaching the cells after differentiation into M1- or M2-like macrophages. 0.1×10^6 cells were differentiated in 48-well plate and different treatments were applied once the differentiation completed.

With M1-like macrophages and after 6 hours of culture, IL-1 β concentration was the highest in R upon LPS and BzATP treatment. IL-1 β concentrations in E+R and E are as low as if the cells were not fully stimulated to release IL-1 β . The same happened upon treatment with LPS and nigericin. P-values below 0.05 and corresponding IL-1 β concentrations after dual stimulation (LPS + BzATP or LPS + nigericin) are summarized in Table 21. Data are visualized in Figure 26A.

Table 21: Select IL-1 β concentrations after 6 hours of culture of M1-like macrophages in different media.

Condition		IL-1 β [pg/ml]	p (R vs bE)	p (R vs E)	p (bE vs E)
LPS + BzATP	R	1906-3232	0.028	0.028	0.833
	E+R	54-159			
	E	37-156			
LPS + nigericin	R	167-2541	0.028	0.028	0.028
	E+R	111-1037			
	E	103-1027			

IL, interleukin; LPS, lipopolysaccharide; BzATP, 2'(3')-O-(4-benzoylbenzoyl)adenosine-5'-triphosphate; R, RPMI-1640 + 10% FBS; E+R, 1:2 mix ECGM-2 + 2% FBS and RPMI-1640 + 10% FBS; E, ECGM-2 + 2% FBS + supplements.

After 20 hours of culture, the overall cytokine concentration decreased. R is still the only medium in which IL-1 β concentrations increased upon treatment with LPS and BzATP/nigericin. P-values below 0.05 and corresponding IL-1 β concentrations after dual stimulation (LPS + BzATP or LPS + nigericin) are summarized in Table 22. Data are visualized in Figure 26B.

Table 22: Select IL-1 β concentrations after 20 hours of culture of M1-like macrophages in different media.

Condition		IL-1 β [pg/ml]	p (R vs bE)	p (R vs E)	p (bE vs E)
LPS + BzATP	R	471-774	0.028	0.028	0.027
	E+R	19-65			
	E	22-54			
LPS + nigericin	R	315-358	0.028	0.028	0.027
	E+R	70-202			
	E	52-137			

IL, interleukin; LPS, lipopolysaccharide; BzATP, 2'(3')-O-(4-benzoylbenzoyl)adenosine-5'-triphosphate; R, RPMI-1640 + 10% FBS; E+R, 1:2 mix ECGM-2 + 2% FBS and RPMI-1640 + 10% FBS; E, ECGM-2 + 2% FBS + supplements.

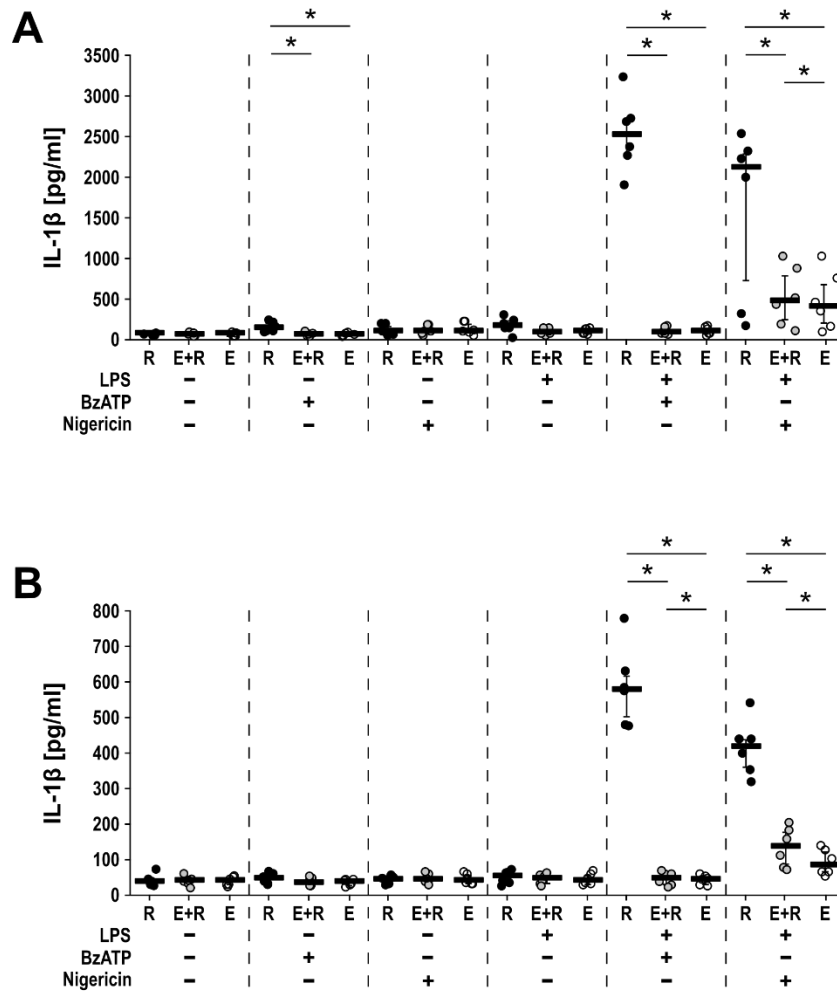


Figure 26: IL-1 β concentration after 6 hours (A) and 20 hours (B) of culture of M1-like macrophages. Cells were left either untreated or stimulated with 1 μ g/ml lipopolysaccharide (LPS) for 5 hours. After priming, 200 μ M 2'(3')-O-(4-benzoylbenzoyl)adenosine-5'-triphosphate (BzATP) or 50 μ M nigericin were applied for 40 minutes. R, RPMI-1640 + 10% FBS; E+R, 1:2 mix RPMI-1640 + 10% FBS and ECGM-2 + 2% FBS + supplement; E, ECGM-2 + 2% FBS + supplements; each dot represents an individual value and bars show the median. Whiskers correspond to the 25th and 75th percentiles, $n=6$, * $p \leq 0.05$. Statistical analysis was performed using the Friedman test, followed by Wilcoxon signed-rank test for pairwise comparisons.

After 6 hours of culture, IL-6 concentrations went up only upon treatment with LPS and in R (2100-3426 pg/ml, Figure 27A). Under the same conditions, IL-6 remained low in E+R (81-369 pg/ml) and E (52-334 pg/ml).

After 20 hours of culture, the profile is similar but with lower concentration (Figure 27B). In R, it only reached 448 pg/ml.

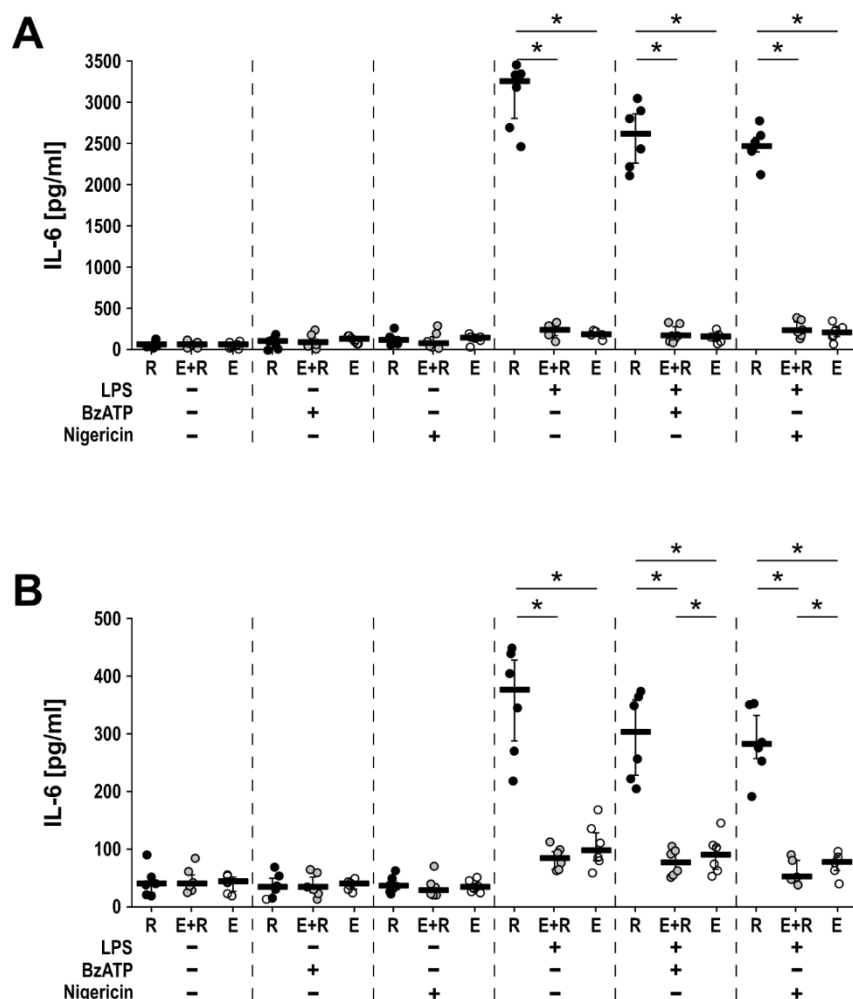


Figure 27: IL-6 concentration after 6 hours (A) and 20 hours (B) of culture of M1-like macrophages. Cells were left either untreated or stimulated with 1 µg/ml lipopolysaccharide (LPS) for 5 hours. After priming, 200 µM 2'(3')-O-(4-benzoylbenzoyl)adenosine-5'-triphosphate (BzATP) or 50 µM nigericin were applied for 40 minutes. R, RPMI-1640 + 10% FBS; E+R, 1:2 mix RPMI-1640 + 10% FBS and ECGM-2 + 2% FBS + supplement; E, ECGM-2 + 2% FBS + supplements; each dot represents an individual value and bars show the median. Whiskers correspond to the 25th and 75th percentiles, n=6, * $p \leq 0.05$. Statistical analysis was performed using the Friedman test, followed by Wilcoxon signed-rank test for pairwise comparisons.

Even though LDH showed important statistically significant variations, there is no pattern showing an increased cell death in a particular medium (Figure 28).

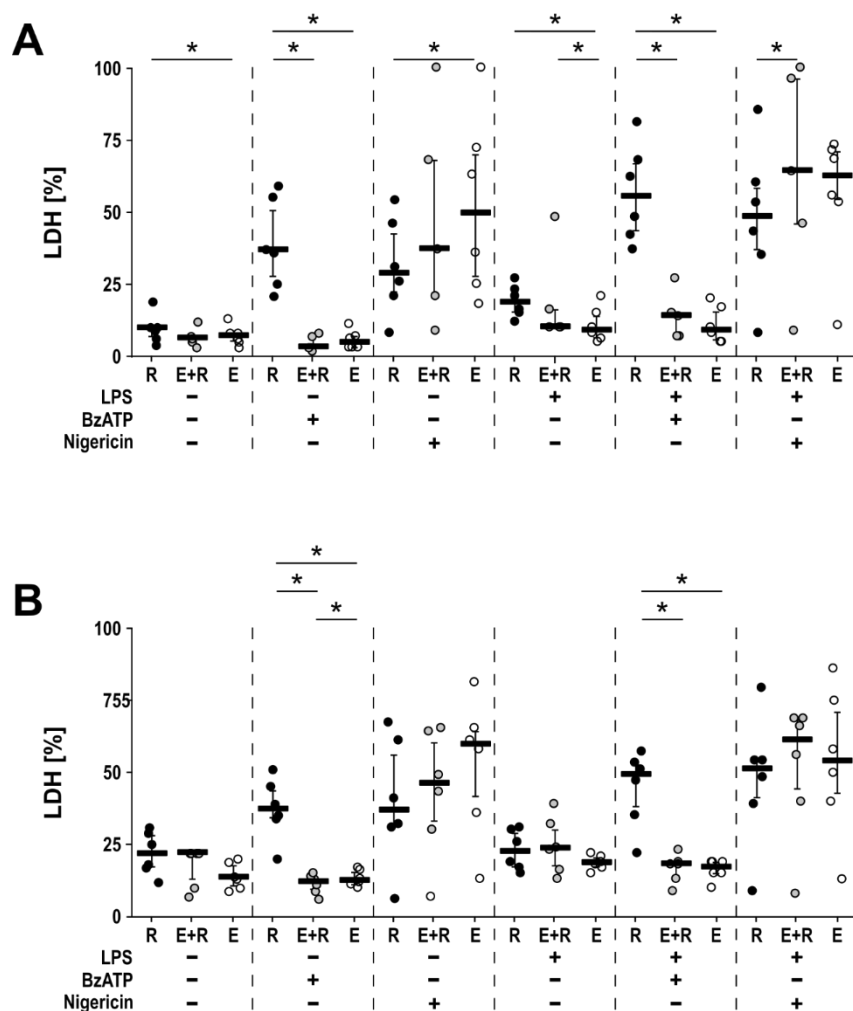


Figure 28: Lactate dehydrogenase (LDH) activity after 6 hours (A) and 20 hours (B) of culture of M1-like macrophages. Cells were left either untreated or stimulated with 1 µg/ml lipopolysaccharide (LPS) for 5 hours. After priming, 200 µM 2'(3')-O-(4-benzoylbenzoyl)adenosine-5'-triphosphate (BzATP) or 50 µM nigericin were applied for 40 minutes. R, RPMI-1640 + 10% FBS; E+R, 1:2 mix RPMI-1640 + 10% FBS and ECGM-2 + 2% FBS + supplement; E, ECGM-2 + 2% FBS + supplements; each dot represents an individual value and bars show the median. Whiskers correspond to the 25th and 75th percentiles, n=6, * p ≤ 0.05. Statistical analysis was performed using the Friedman test, followed by Wilcoxon signed-rank test for pairwise comparisons.

The medium mix E+R showed similar effects on M2-like macrophages than on M1-like macrophages regarding IL-1 β concentrations (Figure 29A). Even though overall concentrations are low in comparison to the M1-like macrophage culture, higher concentrations were measured in R, especially upon treatment with LPS and BzATP/nigericin (9-139 pg/ml). Cytokine concentrations in E+R and E remained low and similar independent of the treatment (7-12 pg/ml with LPS + BzATP, 9-71 pg/ml with LPS + nigericin). After 20 hours of culture of M2-like macrophages, the same concentration profile was observed but with higher overall concentrations (Figure 29B). IL-1 β was always higher in R (79-308 pg/ml upon stimulation with LPS + BzATP/nigericin) and lower, but similar, in E+R and E (4-17 pg/ml with LPS + BzATP, 22-141 pg/ml with LPS + nigericin).

pg/ml), while concentrations in E+R remained between 31 and 77 pg/ml and in E between 11 and 122 pg/ml.

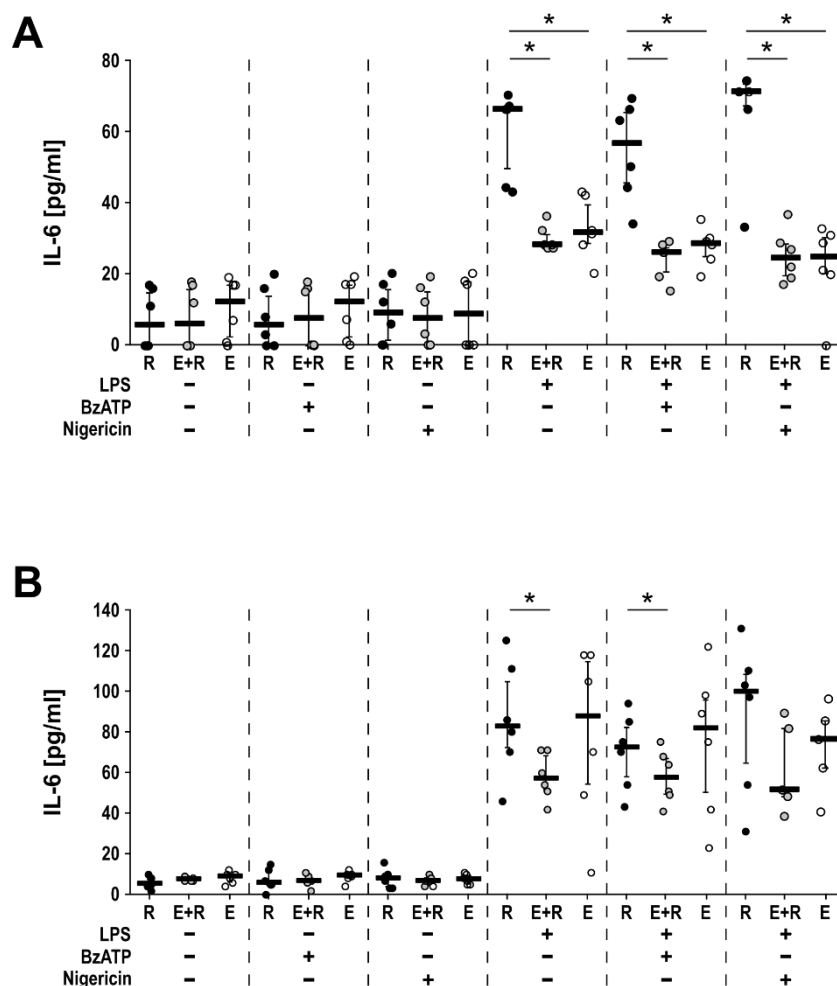


Figure 30: IL-6 concentration after 6 hours (A) and 20 hours (B) of culture of M2-like macrophages. Cells were left either untreated or stimulated with 1 μ g/ml lipopolysaccharide (LPS) for 5 hours. After priming, 200 μ M 2'(3')-O-(4-benzoylbenzoyl)adenosine-5'-triphosphate (BzATP) or 50 μ M nigericin were applied for 40 minutes. R, RPMI-1640 + 10% FBS; E+R, 1:2 mix RPMI-1640 + 10% FBS and ECGM-2 + 2% FBS + supplement; E, ECGM-2 + 2% FBS + supplements; each dot represents an individual value and bars show the median. Whiskers correspond to the 25th and 75th percentiles, n=6, * $p \leq 0.05$. Statistical analysis was performed using the Friedman test, followed by Wilcoxon signed-rank test for pairwise comparisons.

LDH activity was globally stable over the different conditions and media. Only upon treatment with LPS + BzATP, LDH was higher in R (37-81% after 6 hours of culture, 22-57% after 20 hours) than in E+R or E (5-27 after 6 hours, 9-23% after 20 hours) after both 6 and 20 hours of culture (Figure 31A,B).

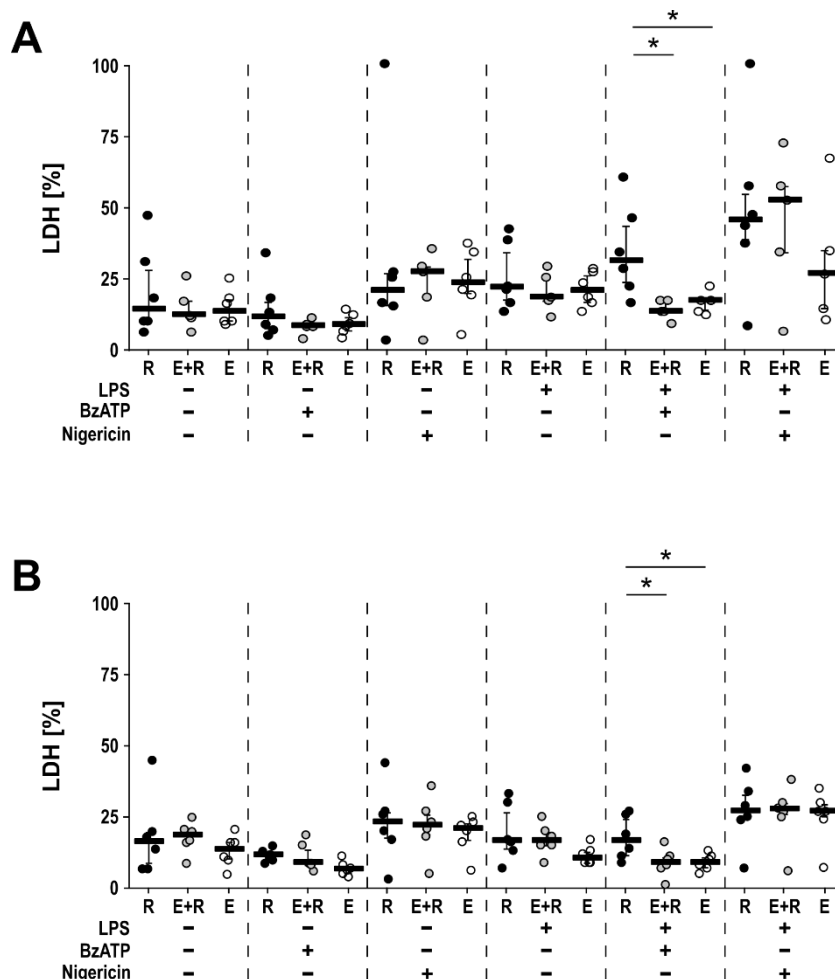


Figure 31: LDH activity after 6 hours (A) and 20 hours (B) of culture of M1-like macrophages. Cells were left either untreated or stimulated with 1 μ g/ml lipopolysaccharide (LPS) for 5 hours. After priming, 200 μ M 2'(3')-O-(4-benzoylbenzoyl)adenosine-5'-triphosphate (BzATP) or 50 μ M nigericin were applied for 40 minutes. R, RPMI-1640 + 10% FBS; E+R, 1:2 mix RPMI-1640 + 10% FBS and ECGM-2 + 2% FBS + supplement; E, ECGM-2 + 2% FBS + supplements; each dot represents an individual value and bars show the median. Whiskers correspond to the 25th and 75th percentiles, n=6, * $p \leq 0.05$. Statistical analysis was performed using the Friedman test, followed by Wilcoxon signed-rank test for pairwise comparisons

3.2.8 Impact of Matrigel and E on macrophages

50,000 THP-1 cell-derived M1- and M2-like macrophages were cultured in 96-well plates and maintained in different media to evaluate their effects on cell morphology (Figure 32) and distribution. The three media were R (RPMI-1640 + 10% FBS), which was the standard medium for macrophages, E (ECGM-2 + 2% FBS + supplements), which was the standard medium for HUVECs. The third medium was a 1:2 mix of the two. Phase-contrast photomicrographs were taken after 18 hours of culture, corresponding to the time required for future angiogenesis assays in co-culture of HUVECs and macrophages (see chapter 2.2.7).

No obvious differences were observed between the three media. M1-like macrophages tended to form small cell clusters in comparison to M2-like macrophages that were evenly distributed.

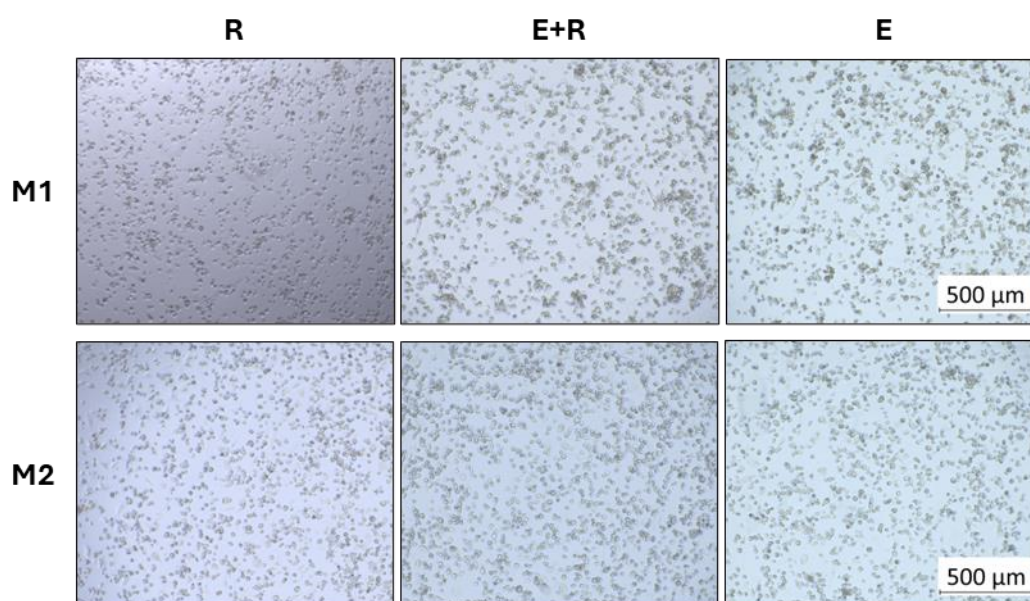


Figure 32: Photomicrographs of THP-1 cell-derived M1- and M2-like macrophages in different media. 50,000 cells in different culture media were seeded on a 96-well plate. One representative passage out of five is shown, phase contrast. R, RPMI-1640 + 10% FBS; E+R, 1:2 mix RPMI-1640 + 10% FBS and ECGM-2 + 2% FBS + supplements; E, ECGM-2 + 2% FBS + supplements.

In parallel, a similar experiment was done on Matrigel-coated plates to evaluate their effect on macrophages (Figure 33). 50,000 M1- and M2-like macrophages were seeded and maintained in the three identical media described before. The M1-like macrophage clusters were bigger when cells were cultured on Matrigel. However, M2-like macrophages stayed the same and distributed well, independent of the culture medium.

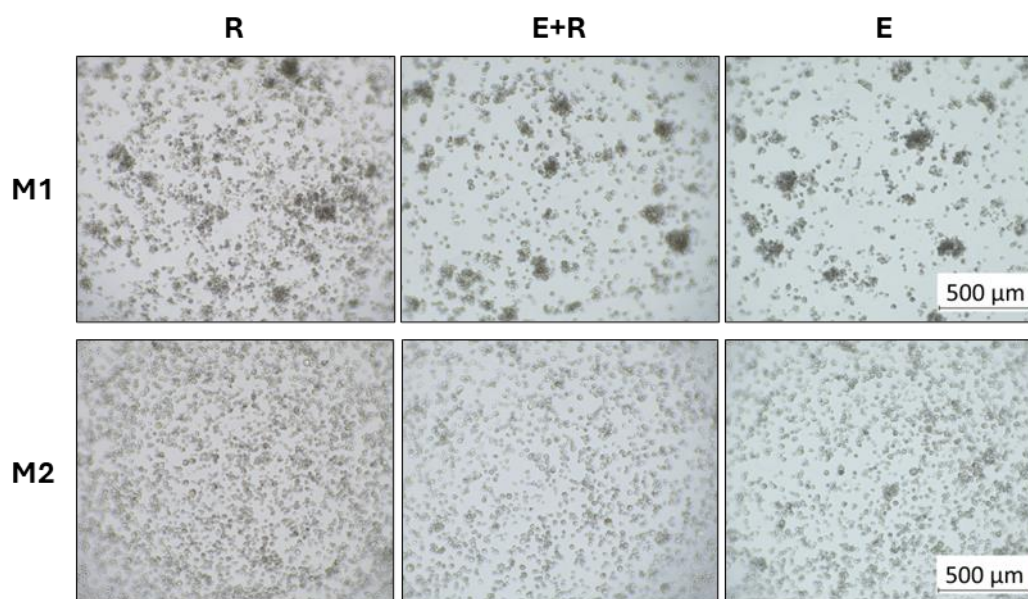


Figure 33: Photomicrographs of THP-1 cell-derived M1- and M2-like macrophages in different media and on Matrigel. 50,000 cells in different culture media were seeded on a 96-well plate coated with Matrigel for 18 hours. One representative passage out of three is shown, phase contrast. R, RPMI-1640 + 10% FBS; E+R, 1:2 mix RPMI-1640 + 10% FBS and ECGM-2 + 2% FBS + supplements; E, ECGM-2 + 2% FBS + supplements.

3.3 Tube formation assay

To quantify the effect of different media (E, bE, ECGM-2 and R) or of different macrophage subtypes (M0-, M1- and M2-like) on tube formation, tube formation assays were performed on Matrigel in 96-well plates followed by counting the number of meshes and segments. HUVECs were incubated 1 hour before the macrophages were added, and then for another 17 hours, after which photos were taken. Meshes and segments were counted on the photomicrographs representing an area of 2.4 mm² (Figure 34).

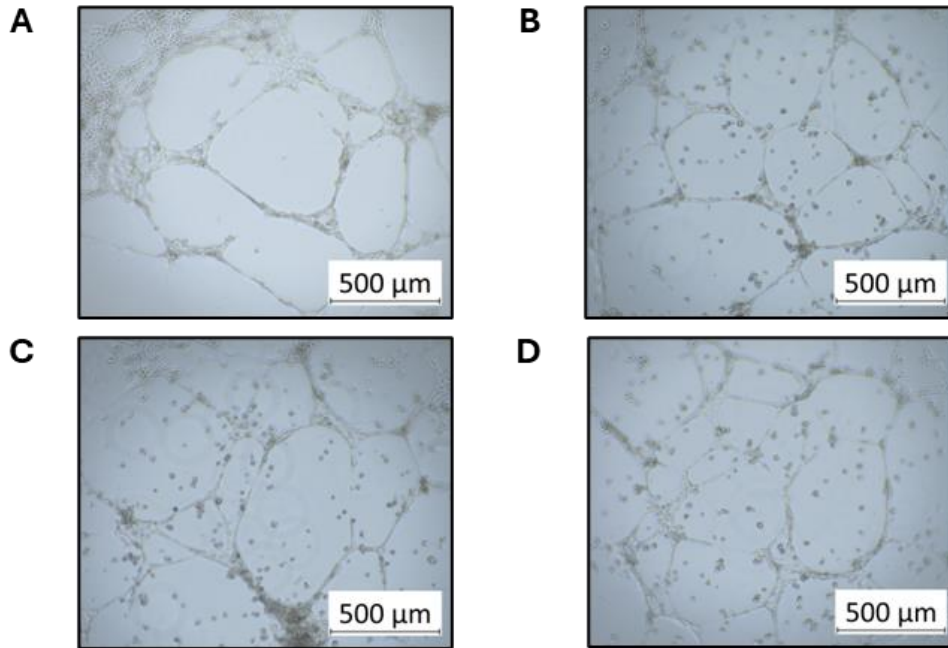


Figure 34: Tube formation assay in mono- or co-culture in E. 10,000 HUVECs were cultured on top of Matrigel in 96-well plates (A). 5,000 M0- (B), M1- (C), or M2-like macrophages (D) were added according to the experimental set-up. Cells were cultured in ECGM-2 + 2% FBS + supplements (E). One representative passage out of five is shown, phase contrast.

When looking at the HUVECs cultured alone, ECGM-2 with or without FBS or supplements did not change the number of meshes (1.25-7.88 per photomicrograph, Figure 35A). Only R led to a significant decrease in mesh formation (0.63-3 per photomicrograph). Within the same media, none of the macrophage subtypes enhanced mesh formation in comparison to HUVEC monocultures. Only in R M1- and M2-like macrophages enhanced mesh formation in comparison to HUVEC monocultures (1.88-4.25 meshes per photomicrograph for the M1-like, and 1.38-3.88 per photomicrograph for the M2-like macrophages). However, there were no significant differences in the improvement between M1- and M2-like macrophages. The number of segments followed almost the same pattern (Figure 35B). In R less segments were counted, when comparing the HUVEC monocultures in R to E (17.88-21.75 segments per photomicrograph in E, 6.50-17.88 per photomicrograph in R). In R, M0-, M1- and M2-like macrophages increased the segment formation in comparison to HUVEC monocultures (6.75-19.50

segments per photomicrograph for the M0-like, 9.88-22.88 per photomicrograph for the M1-like, and 8.00-23.00 per photomicrograph for the M2-like macrophages).

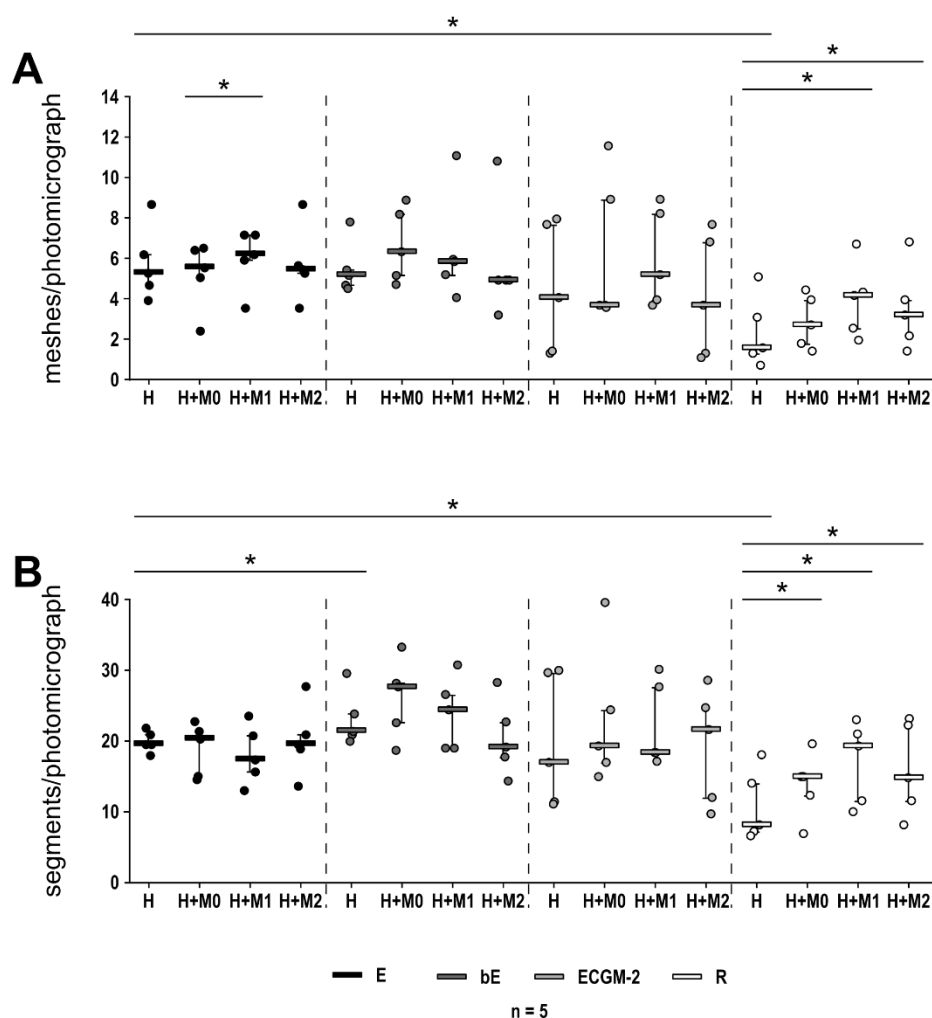


Figure 35: Tube formation assay in different media and in the presence of macrophages. 10,000 HUVECs were cultured on top of Matrigel in 96-well plates. 5,000 macrophages (M0-, M1- or M2-like) were added according to the experimental set-up. Cells were cultured either in ECGM-2 + 2% FBS + supplements (E), ECGM-2 + 2% FBS (bE), ECGM-2 or RPMI-1640 (R). H, HUVECs; M, macrophages. Meshes (A) and segments (B) were counted at the end of the assay on each photomicrographs (2.4 mm²). Each dot represents an individual value calculated out of 4 photos and duplicates. Bars show the median. Whiskers correspond to the 25th and 75th percentiles, n = 5, * p ≤ 0.05. Statistical analysis was performed using the Friedman test, followed by Wilcoxon signed-rank test for pairwise comparisons.

4 Discussion

4.1 Discussion of the methods

4.1.1 THP-1 cells and HUVECs

In this study, macrophages were used in many experiments, either to assess inflammatory potential of printed surfaces, to evaluate the effect of different media on cytokine release and cell viability, or to evaluate their effect on angiogenesis. The macrophages used for those experiments were derived from the monocytic THP-1 cell line after PMA-based differentiation. The THP-1 cell line was isolated in 1980 from the peripheral blood of a one-year-old male patient with acute leukemia and are commonly used to study inflammation *in vitro* (Resto-Ruiz et al., 2002; Chanput et al., 2010; Huang et al., 2021; Jian et al., 2021). While primary cells might better reflect what is happening *in vivo*, using a tumor cell line presents many advantages such as increased proliferation (Tedesco et al., 2018) and less genetic variability (Chanput et al., 2014; Tedesco et al., 2018). Previous studies also showed differences in cytokine secretion and surface markers in comparison to primary cells, that highly depend on the differentiation or stimulation protocols (Daigneault et al., 2010; Tedesco et al., 2018; Giambelluca et al., 2022).

There are countless THP-1 differentiation and polarization protocol in the literature. Recent studies have shown that different protocols result in different surface marker and cytokine expression. PMA is generally used for differentiation from monocyte to M0-like macrophages at concentrations between 2 and 200 ng/ml for 24-72 hours (Mohd Yasin et al., 2022). Various stimuli with different concentrations and different incubation times are also used for the polarization resulting in a high heterogeneity in macrophage behavior (Pinto et al., 2021). This heterogeneity makes it difficult to compare studies. However, the protocol used in this study has been recently fully characterized (Wolf et al., 2025).

The second cell type that was used for angiogenesis experiments were HUVECs originating from the umbilical cord vein of human newborns. Hence, they are primary human EC but still easy to cultivate. HUVECs represent a well-established model to study vascularization. The effect of inflammatory cytokines as well as angiogenic factors on *in vitro* tube formation are well studied. Their sensibility to growth factors or cytokines make them perfectly suitable to study tube-formation (Arnaoutova et al., 2009).

4.1.2 Flow cytometry

The phenotype of macrophages is influenced by many factors (differentiation protocol and chemical environment) and is never stable. M1- or M2-like phenotypes exist in a continuum and show plasticity in response to various stimuli (Forrester et al., 2018). Assessing macrophage phenotypes under different conditions is hence essential to establish their properties during experiments. In this study, macrophage phenotypes were investigated by flow cytometry analysis of surface marker expression on M0-, M1-, and M2-like macrophages. The panel of surface marker for flow cytometry analysis was chosen to cover a wide range of typical M1 and M2 markers. There is no classic panel to characterize macrophages. CD38, CD80, CD83, CD86, and HLA-DR are associated with pro-inflammatory functions and the M1 phenotype as they mediate the activation of T cells and correlate with pro-inflammatory cytokine release. CD163, CD206, and CD209 are associated with the M2-like macrophages, which are promoting anti-inflammatory functions such as wound healing, tissue regeneration, homeostasis and clearance (see chapter 1.3). Theoretically, the M2 phenotype is of interest for our co-culture with HUVECs to promote tube formation (Jetten et al., 2014). However, some studies suggest that the M1 phenotype might play a role in the initiation of tube formation (Spiller et al., 2015; Graney et al., 2020). Therefore, both phenotypes were investigated.

4.1.3 Cytokine release and estimation of cell death

Cytokine concentrations were measured in cell culture supernatants using ELISA. It is important to note that the materials on which cells are cultured (IP-Visio, IP-S, glass, ITO-coated glass or culture plates) can interact with these cytokines and potentially influence their apparent concentration in the supernatant. Studies have shown that cytokines can adsorb to the surface of plastic surfaces used for cell culture, leading to an underestimation of their actual concentration in cell culture supernatants (Shankar & Cohen, 1997; Veerasubramanian et al., 2022). Therefore, the measurement may not accurately reflect the total amount of cytokines released by macrophages but only the current supernatant concentration. If such adsorption occurred, it should be uniform across all experimental conditions and would not affect the comparisons between samples. When differences in cytokine concentrations are observed, it is not possible to determine whether these changes result from modulation of cytokine expression,

secretion or stability. ELISA measurements only provide information on the final cytokine concentration in the supernatant, without indicating the precise underlying mechanism. Moreover, phenol red present in the medium (different from RPMI and ECGM), changes in pH as well as the stimulants added can slightly disturb the readouts. Further, it should be noted that the results of an ELISA do not give any hint on the specificity of the reaction. In contrast, Western blotting gives the molecular mass of the antigen investigated, which can serve as a confirmation of specificity. However, the quantification of antigens by Western blotting is by far less precise compared to ELISAs.

LDH activity was measured to estimate the impact of different parameters on cell death. LDH is a stable cytosolic enzyme that is released into the supernatant when the plasma membrane is damaged. Higher LDH activity reflects increased cell death. However, it is important to note that elevated LDH can correlate with increased IL-1 β concentrations. Mechanical stress (for example due to strong or repeated pipetting) can cause membrane disruption and the release of intracellular material, including IL-1 β or its precursor pro-IL-1 β , which can also be detected by the ELISA, and could explain small cytokine concentrations detected upon cell treatment with LPS only (Herzyk et al., 1992). In addition, activation of the NLRP3 inflammasome and later caspase-1 can induce pyroptosis, a pro-inflammatory form of cell death (Fink & Cookson, 2006). The first signs of pyroptosis appear after 30 minutes to 1 hour (Bai et al., 2024) and promotes inflammation in the surrounding environment through the release of intracellular compounds into the supernatant (W. He et al., 2015). Consequently, increased LDH activity may be associated with an increase in IL-1 β concentrations in the supernatant, potentially affecting the results. In some cases, treatment with nigericin leads to elevated LDH activity, particularly in LPS-primed cells (He et al., 2015; Zhou et al., 2020). In this study, nigericin is applied for only 40 minutes, which should limit the extent of cell death to a moderate level.

According to the provider of the kit for the measurement of LDH activity, LDH has a half-life of approximately 9 hours under standard *in vitro*. However, LDH measurements at a given time point reflect a combination of factors such as ongoing release from cells undergoing recent death, accumulation from cells that died earlier, and partial enzyme degradation over time. Therefore, LDH activity measured after 6 or 20 hours of culture represents cumulative cell death over this period rather than exclusively cell death occurring immediately prior to sampling. Moreover, the composition of media (serum

proteins, pH, phenol red) can influence LDH stability and colorimetric readout. Therefore, measuring the LDH activity in cell culture supernatants only provides an estimation of cell death.

4.1.4 Tube formation assay

The tube formation assay is a well-established *in vitro* technique used to evaluate the impact of several factors on vascularization (Arnaoutova et al., 2009), including the effects of macrophages (Spiller et al., 2014). HUVECs are generally cultivated between 6 and 20 hours on Matrigel, during which the cells are migrating to form tubes, similar to primary vessels (Berndt et al., 2021; DeCicco-Skinner et al., 2014). A wide range of cell concentrations is used in the literature from 30,000 to 40,000 cell per cm² (Kazemi-Lomedasht et al., 2014; Cheng et al., 2017)

Tube formation assays can be used to measure the effects of M0-, M1- or M2- like macrophages on the ability of EC to form interconnected structures *in vitro*. To do so, 10,000 HUVECs were cultured in 96-well plates coated with growth factor reduced (GFR) Matrigel for approximately 18 hours (Arnaoutova et al., 2009; Kazemi-Lomedasht et al., 2014; Lee & Kang, 2018). Those parameters are slightly below optimal incubation times and cell numbers to preserve a margin for potential improvement. Four types of media were used: E (ECGM-2 + 2% FBS + supplements), which is the classic medium suggested to culture HUVEC and perform this assay, bE (ECGM-2 + 2% FBS), ECGM-2, and R (RPMI + 10% FBS). In the literature, supplemented medium is often used as a positive control for optimal tube formation (Arnaoutova et al., 2009; Arnaoutova & Kleinman, 2010). Conversely, using non-supplemented medium (either without FBS or without growth supplements) as a negative control that allows to evaluate the specific effects of macrophages fostering tube formation.

Despite being widely used, this assay has several limitations. Matrigel is a basement membrane matrix isolated from a murine sarcoma (Kleinman & Martin, 2005; Passaniti et al., 2022) . Consequently, it is not fully defined, and its composition might vary between batches, causing differences in network formation. HUVECs are primary cells and might as well present batch to batch differences. Their expression profile, phenotype and behavior can also be affected by repeated passages (Nowak-Sliwinska et al., 2018). Finally, FBS has batch to batch variations that could also interfere with the assay.

The quantification of tube formation is typically based on counting parameters such as the number of segments, junctions, and meshes, or on measuring their segment length, and area of meshes. These analyses can be performed either manually or automatically with the ImageJ plugin “Angiogenesis Analyzer” (Carpentier et al., 2020). In the literature, some studies apply more detailed classifications, for example by distinguishing between master junctions, master segments, twigs, and nodes (Al Halawani et al., 2021). However, in our case the plugin did not provide reliable and consistent results. The presence of macrophages interfered with the identification of endothelial structures, which prevented an accurate automated network analysis. Considering the difficulty in discriminating distinctive features, the analysis was restricted to manually counting meshes and segments as indicators of network quality.

4.2 Macrophage behavior on printed surfaces

4.2.1 PlasCLEAR

IL-1 β and IL-6 concentrations were quantified with or without a PlasCLEAR insert positioned at the bottom of the well plates to evaluate the potential of PlasCLEAR to induce or enhance pro-inflammatory cytokine secretion. LDH activity was also measured to estimate the cell viability and to conclude on PlasCLEAR cytocompatibility. The fact that IL-1 β or IL-6 remained low in the absence of all stimuli required (LPS and BzATP or nigericin for IL-1 β and at least LPS for IL-6) showed that PlasCLEAR did not prime the cells by itself. IL-1 β concentrations were significantly lower in the wells containing a PlasCLEAR insert, when the cells were treated with both LPS and BzATP/nigericin, suggesting an anti-inflammatory potential. PlasCLEAR could either reduce the release of this cytokine from M1-like macrophages or absorb the cytokine more than the well-plate material does. IL-6 concentrations remained at about the same level, irrespective of the presence or absence of PlasCLEAR. The LDH activity remained also low in every condition, which is in line with a high cytocompatibility of PlasCLEAR, as already mentioned in a previous study done by our cooperation partners on EC (Fritschen et al., 2022). Unfortunately, the chemical nature of PlasCLEAR is not given by the resin manufacturer, which does not allow comparison with the current literature on similar surfaces.

4.2.2 IP-Visio and IP-S

The cytocompatibility and inflammatory potential of IP-Visio, IP-S, and ITO (printing substrate) were assessed by measuring the release pro-inflammatory cytokines, evaluating cell adherence and measuring LDH activity. In this experimental set-up, THP-1 cell-derived macrophages were cultured on glass (inert material) as a negative control. Stimulation with LPS combined with BzATP or nigericin to induce IL-1 β release, or LPS (with or without BzATP or nigericin) to induce IL-6 release was used as the positive control. LDH activity, cell morphology, adherence and distribution as revealed by Pappenheim staining were in line with good cytocompatibility of the resins. Cells were as adherent on glass as on IP-Visio, IP-S and even ITO with no clear changes in cell morphology. LDH activity was stable and low among every condition. Only BzATP increased the LDH activity, but the median remained always below 25% for IP-Visio or IP-S, and below 30% for ITO-coated glass. IL-1 β concentrations in the cell culture supernatants of macrophages stimulated with LPS and nigericin were also slightly higher on ITO-coated glass than they were on glass, suggesting a moderate pro-inflammatory potential. However, ITO-coated glass is not the material of interest in this study, but only the substrate used for printing. Since IP-Visio did not provoke the same increase in IL-1 β concentrations under the same condition, it seems that ITO does not interfere with the evaluation of the resins. For this reason, ITO-coated glass has been excluded from the next experiment with IP-S. Upon treatment with LPS and BzATP or nigericin, IP-Visio was not associated with elevated IL-1 β concentrations, it remained at the same level as it was on glass suggesting neither a stronger nor lower inflammatory potential. However, IL-6 concentrations were increased upon treatment with LPS when the cells were cultivated on IP-Visio, this time suggesting slightly pro-inflammatory properties. On the other hand, IP-S was never associated with elevated IL-1 β or IL-6 concentrations. IL-1 β concentrations were significantly lower on IP-S upon treatment with LPS + nigericin. Neither IP-Visio nor IP-S provoked an elevated cytokine concentration when not all the stimuli were added, suggesting that these resins cannot trigger an inflammatory response by themselves.

There are very few studies regarding IP-Visio and IP-S resins. Their chemical nature is also not given by the manufacturer, making it difficult to compare these surfaces with the literature. However, they are both commercialized as biocompatible. Two studies are qualifying IP-Visio and IP-S as cytocompatible as well (Moussi et al., 2020; Rosenbohm

et al., 2022). Both studies used cell lines (A431 and HCT 116) and cultured the cells for a maximum of 24 hours.

4.3 Assessment of macrophage subsets under co-culture conditions

4.3.1 Challenges of the co-culture of HUVECs and macrophages

The second overriding aim of this thesis was to improve vasculogenesis and angiogenesis in artificial tissues using macrophages derived from a cell line. The co-culture of HUVECs and polarized THP-1 cell-derived macrophages is associated with a number of uncertainties that need to be tackled before combining both cell types in tube formation assays. Here, the focus was on macrophages. As tube formation assays take 20 hours, macrophages might change their properties during prolonged cultivation. HUVECs and THP-1 cells are typically kept in different media and it is unknown, if THP-1 cell-derived macrophages change their function upon culture in media containing ECGM-2, which is optimized for ECs. Another factor might be the detachment of the differentiated and polarized macrophages from their cell culture plates, which is an unavoidable step when performing tube formation assays co-culturing HUVECs and macrophages. Flow cytometry was used to investigate possible changes in the expression of cell surface markers in response to prolonged culture. Further, cytokine release experiments were done to investigate effects of prolonged culture, the presence of ECGM-2-containing media and combinations thereof.

4.3.2 Assessment of macrophage surface markers after prolonged culture

Changes in the phenotypes of macrophage after 20 hours of culture in R were assessed by flow cytometry. Different surface markers typical for M1- or M2-like macrophages were analyzed by measuring single markers and their co-expression. Co-expression patterns were chosen to distinguish the “classic” M1 or M2 phenotypes, but also to

investigate potential intermediate phenotypes co-expressing M1 and M2 markers (Smith et al., 2016).

The analysis of the co-expression of cell surface markers confirmed that the polarization of the THP-1 cell-derived macrophages was successful. Most of the cells polarized towards M1-like macrophages had a “classic” M1 phenotype (CD38⁺/CD80⁺, CD38⁺/CD209⁻, and CD206⁻/CD38⁺). The CD38⁻/CD209⁺ and the CD206⁺/CD38⁻ phenotypes were predominantly seen in the population of M2-like macrophages, although they represented only 50 and 15% of the entire M2-like population, respectively. The co-expression of CD86⁺ and CD209⁺ representing an intermediate phenotype confirmed the existence of a phenotypic spectrum in the M2-like macrophage population. Cell surface marker expression patterns of M1- and M2-like THP-1 cell-derived macrophages remained stable with subtle modulation between 0 and 20 hours of culture.

CD83 was weakly expressed by all three macrophage phenotypes, M0-, M1, and M2-like. Moreover, CD163 was expressed at the same level in all three phenotypes. The reason for these unexpected results is unknown and there might have been a technical problem in staining the cells for flow cytometry. Therefore, these markers were not used for further analysis. Similar results were obtained previously by our own research group (Wolf et al., 2025) and in a publication from another research group for CD163 (Genin et al., 2015). A study on primary macrophages showed a decrease of CD163 and CD206 MFI when the cells were detached with enzymatic methods like trypsin (S. Chen et al., 2015). Right after their differentiation (0 h of culture), M1- and M2-like macrophages expressed different markers. CD80 and CD38 were highly expressed by the M1-like macrophages. The other M1-markers such as CD86, HLA-DR were expressed at the same level by M1- and M2-like macrophages. CD209 and CD206 were predominant in M2-like macrophages. After 20 hours of culture, CD38 expression decreased and CD209 expression increased in M1-like macrophages. A lower expression of CD38 would be in line with a lower cytokine release in favor of a stronger phagocytosis with increased CD209 expression. M2-like macrophages expressed more CD206 and HLA-DR suggesting greater phagocytosis and antigen-presenting cell activity. Even though these expression differences were significant, they remained in a similar magnitude. The surface marker analysis revealed subtle changes in their expression after 20 hours of culture in R, which does not exclude functional changes. These results are essential to evaluate the impact of our co-culture experimental set-up on macrophages. Even though

this type of co-culture has been performed by other research group, macrophages are not always characterized or only ahead of the co-culture to prove the differentiation (Mayer & et al., 2011; Han et al., 2021; Kun et al., 2024; Landau et al., 2024). Without a proper characterization, it is difficult to draw any conclusions on whether one or another phenotype promotes tube formation.

M2 macrophages are often divided into sub-groups with M2a, M2b, M2c, and M2d. Even though each sub-group is associated with specific functions, there is still no consensus regarding their surface marker expression (Oates et al., 2022; Mondadori et al., 2023). Therefore, we cannot say whether our macrophages tend toward one or another sub-phenotype.

4.3.3 Cytokine release by macrophages after prolonged culture in different media

The cytokine release analysis is a common method used to evaluate macrophage functions. It is complementary to the surface marker analysis. In this study, we measured IL-1 β and IL-6 to evaluate the pro-inflammatory function of M1- and M2-like macrophages. In contrast to the surface marker analysis, the analysis of the cytokine release showed a clear functional switch, when aspects of the tube formation assays were simulated. The effects of the following aspects were investigated alone and in combination: 1) detaching macrophages from the surface of their cell culture plates, 2) culturing the cells for 6 hours (time needed for the stimulation of cytokine release) or 20 hours (time needed for tube formation assay), and 3) culturing the macrophages in different media. In this part, the amount of cytokines released into the cell culture supernatant is discussed by only looking at stimulated macrophages (cells treated with LPS and BzATP or nigericin for IL-1 β , and all conditions with LPS for IL-6).

Cytokine concentrations in cell culture supernatants were much lower after detaching the macrophages compared to cells that remained in their cell culture plates. M1-like macrophages released less than 50% of the IL-1 β and only 25% of the IL-6 in R after 6 hours of culture. This confirms the hypothesis that enzymatic detachment by trypsin-EDTA impairs cell function (Chen et al., 2015). EDTA chelates divalent cations and prevents Ca²⁺-dependent cell adhesion and trypsin is a serine protease that cleaves cell adhesion molecules, also weakening cell attachment. However, trypsin is not specific to

adhesion molecules and can at the same time damage other surface molecules essential in cell function and viability (S. Chen et al., 2015; Lordon et al., 2024). While some studies suggest that the mechanical stress during the detachment might lead to membrane damage and release of DAMPs inducing cytokine release (Wang et al., 2006), others showed that trypsin can impair the LPS signaling via TLR4 and result in lower cytokine release (Komatsu et al., 2012), which would be in line with our results.

In R, IL-1 β and IL-6 concentrations decreased after 20 hours of culture showing that even in an appropriate medium for the culture of THP-1 cell-derived M1-like macrophages, cytokine release is negatively affected by the culture duration.

In ECGM +2% FBS + supplements (E), IL-1 β and IL-6 concentrations were already extremely low after 6 hours of culture, but still negatively affected by longer culture time. The mixed medium E+R led to similar results than E where cytokines remained very low even after 6 hours of culture. bE showed interesting mixed results. IL-1 β concentrations were different in this medium whether cells were treated with LPS + BzATP or nigericin. Concentrations were intermediate between R and E when cells were treated LPS + nigericin, while they were as low as in E when the cells were treated with LPS + BzATP instead. After 20 hours of culture, IL-1 β concentrations remained low when the cells were treated with LPS + BzATP, but they were higher than in R when they were treated with LPS + nigericin. Interestingly, IL-6 concentrations were similar in bE and R after 6 hours of culture, but higher than in any other media after 20 hours of culture. It is difficult to explain the differences in IL-1 β concentrations depending on the treatment. However, it seems that E and E+R did not sustain cytokine release suggesting a loss of function of the M1-like macrophages. However, concentrations in bE were similar or higher than in R suggesting that this medium may be a good compromise to sustain both M1-like macrophages and HUVECs. From this experiment it is impossible to say whether the medium could cause damage to the cells, inducing a stronger cytokine release. Considering the concentration of growth factors and hydrocortisone in ECGM-2, it is not surprising that M1-like macrophage skew towards a M2-like function. EGF, VEGF and IGF-1 contained in ECGM-2 are associated with tissue repair and angiogenic functions in macrophages (Zhao et al., 2016; Spadaro et al., 2017; Wheeler et al., 2018). Hydrocortisone also tends to dampen inflammation and the release of pro-inflammatory cytokines (Liu et al., 2019). Without any supplement (bE), the M1-like macrophages were

releasing more pro-inflammatory cytokines suggesting that their functions are less impaired than when they were cultured in E.

Cytokine release from M2-like macrophages increased with the culture time. They were releasing more IL-1 β and IL-6 after 20 hours of culture than they did after 6 hours, going in the direction of a functional switch. This could be explained by a positive autocrine feedback by accumulating cytokines. Even without stimuli, M2-like macrophages released small amounts of pro-inflammatory cytokines that might slowly switch them towards a M1-like phenotype (Pelegrin & Surprenant, 2009). Since the medium was changed after the differentiation and before the 20 hours of culture, the hypothesis of stress caused by nutrient deprivation induced by the media is less probable. The observations reported for the M1-like macrophages also applied to the M2-like macrophages even though the overall cytokine concentrations were lower. E and E+R led to a low release of IL-1 β and IL-6 cytokines, which could be expected from the M2-like macrophages. bE again led to similar concentrations than in R after 6 hours of culture, and higher concentrations than R after 20 hours of culture. R was considered as a positive control in our experiments, since macrophages are often cultured with this medium in the literature (Forsythe et al., 1996; Richter et al., 2023). The fact that there is less cytokine release in E and E+R might either suggest that the M2-like macrophages are further polarized towards a more extreme M2 phenotype (especially thanks to growth factors) and hence release less pro-inflammatory cytokines, or that the biosynthesis of pro-inflammatory cytokines was inhibited.

In general, we observed wide variations in the concentrations of cytokines and surface markers. These variations might make the results more difficult to interpret. THP-1 cells regularly display high variations between passages.

4.4 Impact of macrophages on tube formation

To assess the potential angiogenic effect of M0-, M1-, and M2-like macrophages on HUVECs, co-culture experiments were performed on Matrigel. HUVECs were seeded upon the Matrigel to let them settle and adhere and macrophages were added 1 hour later. This co-culture was performed in four different media: E (ECGM + 2% FBS + supplements), bE (ECGM + 2% FBS), ECGM-2, and R (RPMI-1640 + 10% FBS). The first one was used as positive control where HUVEC are supposed to perform good tube

and network formation. The two other types of ECGM-based media were sub-optimal, in which HUVECs were supposed to be less sustained by supplements or FBS and therefore forming less tubes and poorer networks. Macrophages could potentially provide the missing factors and improve the results of the tube formation assay. Finally, R was used as negative control and to see if macrophages improved tube formation when kept in their classic culture medium. The number of meshes and segments were counted on each photomicrograph (2.4 mm²) to quantify tube and network formation.

The results of these experiments suggested that HUVECs were not sensible to the lack of FBS or supplements. The number of meshes and segments remained similar whether the HUVECs were cultured in all three types of ECGM-2-containing media. The addition of any subtype of macrophages did not make a difference either. However, in R, HUVECs formed less meshes and less segments. This time, M1- and M2-like macrophages enhanced mesh and segment formation. Even M0-like macrophages enhanced segment formation.

Those results were very surprising when compared to the literature where macrophages, and especially the M2-like macrophages, are described as pro-angiogenic (Jetten et al., 2014). However, for the studies really performing either direct co-culture or using transwell culture systems (and not using conditioned medium), none are characterizing the phenotype and functional properties of the macrophages and only few of them detail the culture media (Jetten et al., 2014; Han et al., 2021; Kun et al., 2024). Moreover, growth factor-free ECGM is commonly used as negative control for tube formation assay, while the complete ECGM is used as positive control (Arnaoutova & Kleinman, 2010; Sarkanen et al., 2011; Martinez-Sanchez et al., 2019). In our hands, the presence of FBS and growth factors had no impact on tube formation. In these media macrophages did not have any impact either, suggesting that tube formation cannot be improved and is already at its best. The fact that M1- and M2-like macrophages improve tube formation in R in the same way is consistent with the analysis of cytokine release, as the two phenotypes seemed to converge toward similar functions during prolonged cultivation. Different batches of medium, FBS, growth factors, Matrigel and HUVECs were used during these experiments to minimize the possibility of batch-dependent errors. However, it could lead to greater variations in the results, erasing subtle variations. Although bias during the counting cannot be completely excluded, it was limited by not knowing the experimental conditions. It was impossible to identify the culture medium or the macrophage

phenotype by looking at the photomicrographs. Only the monoculture of HUVEC was visually different from the co-culture.

5 Conclusions

The analysis of IL-1 β and IL-6 release by THP-1 cell-derived macrophages on IP-Visio, IP-S and PlasCLEAR showed that these resins presented a neutral inflammatory profile and only caused subtle modulation of macrophage function. Without any treatment, M1-like macrophages did not secrete a large amount of cytokines. When macrophages were pre-treated to stimulate cytokine release, they did not over-express these cytokines. Both results suggest that these surfaces do not induce inflammation. IP-Visio, IP-S and PlasCLEAR were tested regarding their pro- or anti-inflammatory potential for the first time, and they seem to be promising candidates to print vascular networks for use in the biomedical field. The adhesion of the cells to the surfaces and the good viability of the macrophages were in line with the cytocompatibility of these resins, which was already described before. Of course, to state biocompatibility of artificial surfaces, more tests must be performed. It would be important to consider more cytokines, to investigate the surfaces under flow conditions, to measure a larger panel of cytokines, and to perform experiments *in vivo*.

In the second part of the study, THP-1 cell-derived M1- and M2-like macrophages were characterized in the perspective of co-culturing them with HUVECs to sustain vasculogenesis in artificial tissues. We simulated the conditions of tube formation assays and saw that the prolonged time of culture typical for these assays essentially did not change the expression of macrophage surface markers. However, prolonged cultivation, cell detachment, and the use of media suitable for the culture of HUVECs resulted in a reduced release of pro-inflammatory cytokines by M1-like macrophages and in increased cytokine secretion by M2-like macrophages. This suggests that the functional properties of M1-like and M2-like macrophages are converging. In co-culture with HUVECs, M0-, M1- and M2-like macrophages seemed to enhance network formation only in R. This medium is suited to culture macrophages but not HUVECs. Whenever an ECGM-2-containing medium was used, tube formation was not enhanced in the presence of any subtype of macrophages. Based on these results, it is not possible to answer the question of whether THP-1 cell-derived macrophages can improve blood vessel formation.

Summary

English

Biohybrid organs are limited in size by the lack of a vascular network, which could be overcome by a network produced by two-photon polymerization. The lumina of these networks could be coated with endothelial cells. In this context, macrophages are relevant, because they mediate foreign body reactions and can support the formation of blood vessels. Macrophages form a continuum of different phenotypes with pro-inflammatory M1 and anti-inflammatory M2 macrophages being the extremes. The first aim of this study was to assess the biocompatibility of the printing resins IP-Visio and IP-S. The second aim was to investigate the potential of macrophages derived from a monocytic cell line to foster blood vessel formation.

M1-like macrophages were differentiated out of THP-1 cells and cultured on printed IP-Visio and IP-S in the absence or presence of appropriate stimuli that induce the release of the pro-inflammatory cytokines, interleukin (IL)-1 β and IL-6. Before performing tube formation assays, these assays were simulated using M0-, M1-, and M2-like THP-1-derived macrophages to assess potential changes. Thereafter, tube formation assays were performed using endothelial cells in co-culture with macrophages.

M1-like macrophages released cytokines in response to appropriate stimuli. IP-Visio or IP-S did not further stimulate cytokine release. The resins allowed for a proper adhesion of macrophages and did not induce cell death. Simulation of tube formation assays in a medium compatible with the culture of macrophages led to subtle changes in cell surface marker expression and to a reduction of the cytokine release by M1-like macrophages, while that of M2-like macrophages increased. The cytokine release was drastically reduced when macrophages were cultured in endothelial cell culture media. M1- and M2-like macrophages only improved the results of the tube formation assays, when the co-culture was performed in a medium typically used with macrophages.

The results of this study are consistent with the assumption that IP-Visio and IP-S are cytocompatible and do not provoke inflammation, making them promising materials for vascular network printing. No firm conclusions can be drawn from the tube formation assays. Additional research is needed to decide if THP-1 cell-derived macrophages can promote the vascularization in engineered tissues.

German

Biohybride Organe sind in ihrer Größe durch das Fehlen von Gefäßen begrenzt, was durch ein per Zweiphotonendruck hergestelltes Gefäßnetzwerk gelöst werden könnte. Die Lumina solcher Netzwerke könnten mit Endothelzellen besiedelt werden. In diesem Zusammenhang sind Makrophagen bedeutsam, da sie Fremdkörperreaktionen vermitteln und die Bildung von Blutgefäßen unterstützen können. Makrophagen bilden ein Kontinuum verschiedener Phänotypen, wobei die proinflammatorischen M1- und die antiinflammatorischen M2-Makrophagen die Extreme darstellen. Das erste Ziel dieser Studie war, die Biokompatibilität der Kunstharze IP-Visio und IP-S zu beurteilen. Das zweite Ziel war, die Förderung der Blutgefäßbildung durch Makrophagen zu untersuchen, die von einer Zelllinie abstammen.

M1-ähnliche Makrophagen wurden aus THP-1-Zellen differenziert und auf gedrucktem IP-Visio und IP-S in Abwesenheit oder Anwesenheit von Stimuli kultiviert, die die Freisetzung der proinflammatorischen Zytokine Interleukin (IL)-1 β und IL-6 induzieren. Vor der Durchführung von Gefäßbildungstests wurden diese mit M0-, M1- und M2-ähnlichen aus THP-1-Zellen stammenden Makrophagen simuliert, um potenzielle Veränderungen zu beurteilen. Danach wurden Gefäßbildungstests mit Endothelzellen in Kokultur mit Makrophagen durchgeführt.

M1-ähnliche Makrophagen setzten Zytokine als Reaktion auf entsprechende Stimuli frei. IP-Visio oder IP-S stimulierten die Zytokinfreisetzung nicht weiter. Die Harze ermöglichten eine adäquate Adhäsion der Makrophagen und induzierten keinen Zelltod. Die Simulation der Gefäßbildungstests in einem mit Makrophagen kompatiblen Medium führte zu subtilen Änderungen der Zelloberflächenmarker und zur Reduktion der Zytokinfreisetzung durch M1-ähnliche Makrophagen, während die der M2-Makrophagen anstieg. Die Zytokinfreisetzung war drastisch reduziert, wenn die Makrophagen in Endothelzellmedien kultiviert wurden. M1- und M2-ähnliche Makrophagen verbesserten die Gefäßbildung nur dann, wenn Makrophagenmedium verwendet wurde.

Die Ergebnisse stimmen mit der Annahme überein, dass IP-Visio und IP-S zellkompatibel sind und keine Entzündungen hervorrufen, was sie zu vielversprechenden Materialien für den Druck vaskulärer Netzwerke macht. Die Angiogenesetests erlauben keine sicheren Schlussfolgerungen. Zusätzliche Forschung ist nötig, um zu entscheiden, ob von THP-1-Zellen abgeleitete Makrophagen die Vaskularisierung künstlicher Gewebe fördern.

Abbreviations

2PP	2-photo polymerization
bE	basal endothelial cell growth medium-2 (ECGM-2 + 2% fetal bovine serum)
BzATP	2'(3')-O-(4-benzoylbenzoyl)adenosine-5'-triphosphate
CD	cluster of differentiation
DAMP	damage-associated molecular receptor
DLP	digital light printing
E	endothelial cell growth medium-2 + 2% fetal bovine serum + supplements
EC	endothelial cell
ECGM-2	endothelial cell growth medium-2
ECM	Extracellular matrix
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
GFR	growth factor reduced
HLA	human leukocyte antigen
HRP	horseradish peroxidase
HUVEC	human umbilical vein endothelial cell
IFN	interferon
IL	interleukin
ITO	indium tin oxide
LDH	lactate dehydrogenase
MAPK	mitogen-associated protein kinase
MFI	mean fluorescence intensity
NF	nuclear factor
NLR	nucleotide-binding oligomerization domain-like receptor
NLRP3	NLR family pyrin domain-containing protein 3
OD	optical density
PAMP	pathogen-associated molecular receptor
PDMS	polydimethyl-siloxane
PenStrep	penicillin streptomycin
PMA	phorbol 12-myristate 13-acetate
R	RPMI-1640 + 10% fetal bovine serum
ROS	reactive oxygen species

RPMI-1640	Roswell-Park-Memorial-Institute
PRR	pattern recognition receptor
RT	room temperature
TLR	toll-like receptors
TNF	tumor necrosis factor
TPS	two-photon stereolithography

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Publications

Congresses

Laura Teppe, Katrin Richter, Anna Fritschen, Alina Filatova, Veronika Grau, Ulrike Nuber. *Biocompatibility of printed IP-Visio and interaction of endothelial cells and macrophages.*

International Giessen Graduate Centre of the Life Sciences, 2024 **(Poster)**.

Laura Teppe, Katrin Richter, Anna Fritschen, Alina Filatova, Veronika Grau, Ulrike Nuber. *Biocompatibility of printed IP-Visio artificial vascular networks with macrophages and endothelial cells.*

Science Day, Fachbereich Medizin, Justus-Liebig-Universität Gießen, 2024 **(Poster)**.

Laura Teppe, Katrin Richter, Anna Fritschen, Alina Filatova, Veronika Grau, Ulrike Nuber. *Biocompatibility of printed IP-Visio and macrophage-endothelial cell interactions in blood vessel formation.*

Mini Symposium, Technische Universität Darmstadt, 2024 **(Poster)**.

Laura Teppe, Katrin Richter, Johanna Vetter, Anna Fritschen, Veronika Grau, Ulrike Nuber. *Evaluation of the inflammatory properties of IP-Visio- and IP-S-printed surfaces*

Chirurgische Forschungstage, Bonn, 2025 **(Talk)**.

Laura Teppe, Katrin Richter, Johanna Vetter, Anna Fritschen, Veronika Grau, Ulrike Nuber. *Evaluation of the inflammatory properties of IP-Visio- and IP-S-printed surfaces.*

International Giessen Graduate Centre of the Life Sciences, 2025 **(Poster)**.

Laura Teppe, Katrin Richter, Johanna Vetter, Anna Fritschen, Veronika Grau, Ulrike Nuber. *Evaluation of the inflammatory properties of IP-Visio- and IP-S-printed surfaces.*

Science Day, Fachbereich Medizin, Justus-Liebig-Universität Gießen, 2025
(Talk).

Publications

Wolf, P. M. K., Hanke, D., Singh, V. K., Keller, H. L., Ettischer, L. J., **Teppe, L.**, Amati, A.-L., Hecker, A., Husain-Syed, F., Rohde, M., Nuber, U. A., Büttner, K., McIntosh, J. M., Liese, J., Mazurek, S., Grau, V., & Richter, K. (2025). *The ATP-mediated cytokine release by macrophages is down-modulated by unconventional $\alpha 9^*$ nicotinic acetylcholine receptors.* *Frontiers in Immunology*, 16. <https://doi.org/10.3389/fimmu.2025.1661114>

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Ehrenwörtliche Erklärung

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