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Extracellular Vesicles of Primed Adipose Derived Stem Cells as a Therapeutic Approach for Osteoarthritis

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Zusammenfassung

Einleitung: Osteoarthritis (OA) ist eine komplexe Gelenkerkrankung, die mit Knorpelabbau, Entzündung der Synovialis und Umbau der Knochenstruktur einhergeht. Angesichts der steigenden Prävalenz von OA, insbesondere in der alternden Bevölkerung, sind innovative Strategien zur Bekämpfung von Entzündungen und Gelenkschäden erforderlich. Ein Forschungsansatz für die Therapie der OA ist die Verwendung mesenchymaler Stammzellen (MSC), einschließlich der aus Fettgewebe gewonnener Stammzellen (adipose derived stem cells; ASC). Eine entzündete Umgebung scheint die immunmodulatorischen Fähigkeiten, insbesondere die antiinflammatorische Wirkung von MSC zu verstärken. Das MSC-Sekretom, speziell extrazelluläre Vesikel (EV), spielt bei diesem therapeutischen Ansatz eine entscheidende Rolle. EV sind membrangebundene Partikel, die von Zellen freigesetzt werden, keinen Zellkern haben und eine zellfreie Therapieoption darstellen.

Zielsetzung: Ziel dieser Studie war es, den Einfluss der Zytokine Interferon gamma (INF-γ) und Tumor-Nekrose-Faktor-alpha (TNF-α) auf ASC, ihr Sekretom und EV zu untersuchen. Darüber hinaus wurde der Einfluss von EV, die von stimulierten ASC stammen, auf Knorpelzellen untersucht.

Methoden: ASC wurden 48 Stunden lang mit 25ng/ml TNFα + 25ng/ml INFγ stimuliert. Die EV wurden mit einer modifizierten PEG-Methode mit zusätzlicher Filtration und Volumenkonzentration aus dem konditionierten Medium gewonnen. Zur Analyse der ASC wurden unter anderem die quantitative Polymerase-Kettenreaktion (qPCR), Annexin V/PI-Färbung eingesetzt. Nanopartikel-Tracking-Analyse (NTA) und Durchflusszytometrie wurden verwendet, um EV zu charakterisieren und Unterschiede im Vergleich zur Kontrollgruppe, EV von nicht stimulierten ASC festzustellen. Nachfolgend wurden Knorpelzellen mit EV behandelt, und die Auswirkungen auf genetischer und sekretorischer Ebene wurden mittels qPCR und ELISA-Assay analysiert.

Ergebnisse: Mit der modifizierten PEG-Methode wurden EV erfolgreich angereichert. Das Priming der ASC mit INFγ und TNFα wirkte sich nicht auf die Lebensfähigkeit der Zellen aus, beeinflusste aber die Zellmorphologie erheblich und zeigte eine Tendenz zur Förderung der Proliferation und einen anti-apoptotischen Effekt. Das ASC-Priming hatte keinen nennenswerten Einfluss auf die EV-Konzentration, die Größe oder die exprimierten Oberflächenmarker. Des Weiteren zeigte sich nur in den Genen BCL2 und CCND1 ein Unterschied in der Auswirkung auf Chondrozyten zwischen EV von stimulierten ASC und nicht stimulierten ASC. Wobei gezeigt wurde, dass EV von stimulierten ASC Zellstress in Knorpelzellen induzierten.

Abstract

Introduction: Osteoarthritis (OA) is a complex joint disorder involving cartilage degradation, synovial inflammation, and bone structure remodelling. With the rising prevalence of OA, especially in the aging population, innovative strategies are necessary to address inflammation and joint damage.

One extensively researched approach is the therapeutic use of mesenchymal stem cells (MSC), including adipose-derived stem cells (ASC). Evidence suggests that an inflamed environment enhances the immunomodulatory capabilities of MSC, particular the anti-inflammatory effects. The MSC secretome, particularly extracellular vesicles (EV), plays a crucial role in this therapeutic approach. EV are membrane-bound particles released from cells, lacking a nucleus and offering a cell-free therapy option.

Objective: This study aimed to explore the impact of the cytokines interferon gamma (INFγ) and tumor necrosis factor-alpha (TNFα) on ASC, their secretome, and EV. Additionally, the influence of EV derived from primed ASC on chondrocytes was examined.

Method: ASC were stimulated with 25ng/ml TNFα + 25ng/ml INFγ for 48 hours. EV were separated using a modified PEG method, with additional filtration and volume concentration, from conditioned medium. Various techniques, including quantitative polymerase chain reaction (qPCR), Annexin V/PI staining, and morphological analysis, were employed to analyze ASC. Nanoparticle Tracking Analysis (NTA) and Flow Cytometry were established to characterize EV and identify differences compared to a control. Chondrocytes were then treated with EV, and effects at the genetic and secretory levels were analyzed using qPCR and ELISA assay.

Results: The modified PEG method successfully separated EV from the conditioned medium. Priming ASCs with 25ng/ml TNFα + 25ng/ml INFγ did not impact cell viability but significantly influenced cell morphology, showing a trend toward promoting proliferation and an anti-apoptotic effect. ASC priming had no noteworthy impact on EV concentration, size, or expressed surface markers. Furthermore, a difference in the effects on chondrocytes was only observed in the genes BCL2 and CCND1 between EV from stimulated ASC and not stimulated ASC. It was demonstrated that EV from stimulated ASC induce cellular stress in chondrocytes.

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List of Abbreviations

ASC	Adipose derived stem cells
ECM	extra cellular matrix
EV	Extracellular Vesicles
INFγ	Interferon gamma
MSC	Mesenchymal stem cells
MVB	Multivesicular bodies
NTA	nanoparticle tracking analysis
OA	Osteoarthritis
PEG	Polyethlyenglycol
qPCR	quantitative PCR
TLR	toll like receptors
TNFα	Tumour necrosis factor alpha

1. Introduction

1.1 Osteoarthritis

1.1.1 Definition and Diagnostics

Osteoarthritis (OA) is a heterogeneous condition affecting an entire joint, involving the degradation of cartilage, inflammation of the synovium and remodeling of bone structures (Kolasinski et al., 2020). This complex disorder can be defined radiographically (Kellgren et al., 1957), clinically (Altman, 1991) or based on presenting symptoms. Defining OA as a general illness is complex, given that only half of the patients with radiological indications present symptoms (Bijlsma et al., 2011,Kraus et al., 2015). Concerning the primary cause of OA it can be categorized into primary OA and secondary OA. Primary OA is the most common form, diagnosed in the absence of injuries or trauma, but can be associated with existing risk factor. Secondary OA is linked to preexisting joint abnormality. The cause of these abnormality can be divers, with the most common factors being trauma, injury and congenital disorders (Krishnan et al., 2018,Donahue, 2018).

OA is a progressive condition, leading to pain, stiffness, swelling and loss of joint function. The most affected synovial joints are knees, hips and hands (Kolasinski et al., 2020).

1.1.2 Epidemiology

OA is a common disorder, that has been on the rise over the past years. Currently, it affects approximately 3,3% to 3,6% of the global population (Sen et al., 2023). The lifetime risk of symptomatic OA is around 14%, varying from 9,6% for non-obese males to 23,8% for obese females (Losina et al., 2013). For hand OA, this risk it is even higher, at 39,8% (Qin et al., 2017). This has strong societal implications; for instance, it was the second most expensive disease in the United States in 2011 with a cost of $15 billion (Sen et al., 2023). In Germany, the incidences of coxarthrosis increased by 13,1% from 2009 to 2019 (Walter et al., 2023).

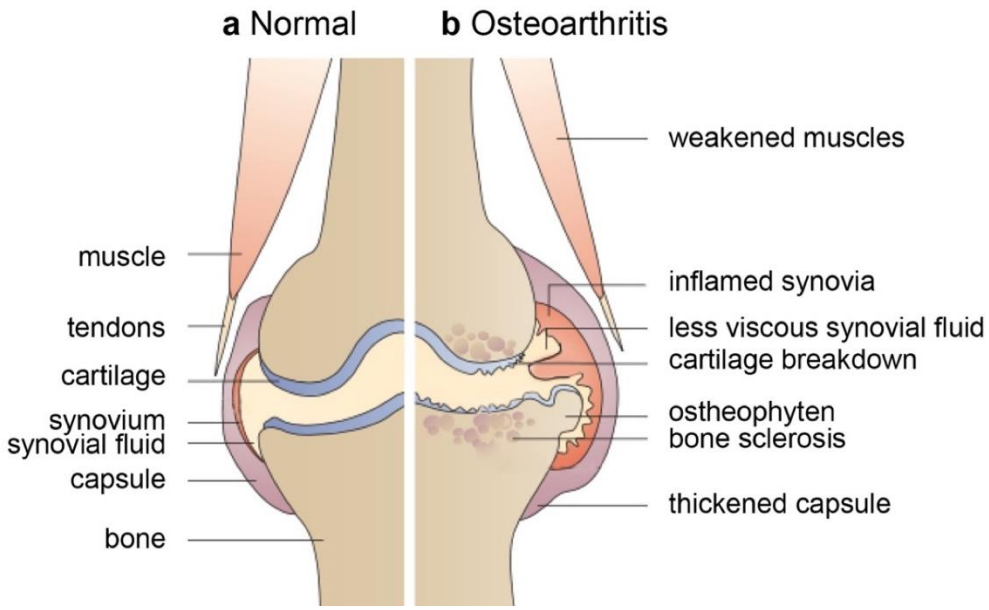

Figure 1: ***Simplified representation of a joint***

a) normal joint b) altered joint due to osteoarthritis with inflamed synovia and cartilage and bone degeneration. Figure is adapted and modified by (Wieland et al., 2005)

1.1.3 Pathogenesis

The pathogenesis of OA has been in focus of researches for decades. Due to the complexity and multiple contributing factors, certain aspects leading to the joint degradation remain unclear.

However, there are risk factors that play a key role both in the initial stages and the progression of the disorder. These factors include age, female gender, obesity, anatomical conditions, muscle weakness, and joint injury (Sen et al., 2023).

In addition to the risk factors, on a cellular level, healthy chondrocytes are crucial to keep non-vascularised cartilage functional and robust, by producing large amounts of extra cellular matrix (ECM). The attempt to repair damaged chondrocytes at an early onset of OA, subsequently fails and leads to an imbalance of cartilage metabolism (Bijlsma et al., 2011, Wieland et al., 2005). This process activates the immune system and together with the secretion of multiple molecules, subsequently results in an inflamed environment (Sohn et al., 2012, Homandberg et al., 1996). Another aspect involves the inflammation of the synovia, which may promote or even initiate the local inflammatory condition in the joint (Sokolove et al., 2013).

In summary, OA is a multifactorial disorder affecting the entire joint, resulting in cartilage degradation, ECM breakdown, local inflammation and subsequent bone destruction.

1.1.4 Therapy Options

OA is currently considered an irreversible condition. Therefore, the main goals of therapy are pain relief, improved mobility and preventing OA progression (Bijlsma et al., 2011).

The existing therapy options can be categorized into (1) non-pharmacological, including life style changes, such as weight loss, individual exercises, as well as thermotherapy; (2) pharmacotherapy, involving topical, oral or intraarticular approaches; and (3) joint replacement (Arden et al., 2021, Falah et al., 2010).

Effective treatments for the underlying causes of OA have not yet been established. However, for many years, several approaches have been addressing this topic. For example, in terms of the chondral defects, bioscaffolds and chondroinductive growth factors have been a main focus in research (Willers et al., 2003). Moreover, the use of mesenchymal stem cells, in particular, appears to have a strong potential in the field of regenerative medicine.

1.2 Mesenchymal Stem Cells

1.2.1 Definition

Stem cells are generally defined as undifferentiated cells with three key characteristics. (1) colony formation, originating from a single cell; (2) potency, the ability to differentiate into various cell types and tissue; and (3) self-renewal, the capability to proliferate.

Stem cells can be classified as totipotent,pluripotent, multipotent and oligopotent. Totipotente stem cells are the least differentiated cell type and occure in the early stage of human development. They can differentiate into both embryonic and extraembryonic tissues. Pluripotent cells can differentiate into tissue from all three germ layers (endoderm, mesoderm, and ectoderm). Multipotent stem cells differentiate into tissue from only one germ layer, while oligopotent stem cells are tissue resident cells, and can form into cells from this specific tissue (Kolios et al., 2013, Ratajczak et al., 2012).

Mesenchymal stem cells are multipotent stem cells, thus can form into tissue from the mesoderm: bone, cartilage, adipose tissue and muscle. These adult stem cells can be derived from variable tissue, such as bone marrow, adipose tissue, Wharton’s jelly, umbilical cord blood and peripheral blood (Augello et al., 2010). In addition to the general stem cell characteristics, MSC are defined by three criteria: (1) they adhere to plastic under normal culture conditions (2) they express

various surface markers such as CD105, CD73 and CD90 while lacking expression of CD45, CD34, CD14 and HLA-DR. (3) they can form into chondroblasts, osteoblasts or adipocytes (Prockop, 1997a).

1.2.2 Origin and Isolation

For a long time, the primary tissue source for MSC was bone marrow. However, over the years, MSC have been identified in various other tissues, including adipose tissue, umbilic cord blood, dental pulp, and synovial fluid (Vats et al., 2005). Even though, MSC from different tissue show a phenotypic heterogeneity and different growth abilities, they full fill the established MSC criteria.

MSC from the adipose tissue offer several advantages, including easy accessibility, a safer harvesting approach, and larger amounts of MSC (Frese et al., 2016, Webb et al., 2012). Adipose derived stem cells can be harvest through liposuction without disturbing the MSC characteristic (Taha et al., 2020).

1.2.3 Secretome

The secretome can be defined as the complex set of all molecules secreted from one cell type. This includes growth factors, cytokines, chemokines, ECM molecules, and extracellular vesicles (Madrigal et al., 2014a, Han et al., 2022). This paracrine activity has been demonstrated to influence both the cell-mediated and humeral immune system and therefor plays a key role in pathological processes (Siegel et al., 2009, Gnecchi et al., 2006). The MSC secretome is strongly influenced by external stimuli, such as hypoxia, cytokines and culture conditions (Madrigal et al., 2014a).

1.2.4 Therapeutic Approach

The ability to differentiate into mesenchymal tissue, the low immunogenicity, and the strong influence of the secretome, makes MSC highly promising for clinical applications (Kolios et al., 2013).

Considering the ability of MSC to form into osteoblasts and chondroblasts, they have been widely utilized in research within the field of bone and cartilage repair (Cheng et al., 2009, Yoon et al., 2007, Grottkau et al., 2013).

Additionally, many studies demonstrated significant results only using the conditioned medium of MSC, suggesting that the secretome of MSC provides a cell free therapy option for inflammatory arthritis (Kay et al., 2017, van Buul et al., 2012, Platas et al., 2013).

Moreover, ASC have been employed to investigate their impact on cardiovascular tissue regeneration, nerve repair and tendon regeneration (Dai et al., 2016, Erba et al., 2010, Chen et al., 2015).

Despite all those promising results, it is crucial to acknowledge that self-renewal capacity of stem cells could potentially lead to carcinogenesis in the host tissue (Filip et al., 2008).

1.3 Priming of Mesenchymal Stem Cells: The Role of TNFα and INFγ

The beginning of an inflammatory setting, as well as in the early stages of tissue destruction and apoptosis are recognized through toll like receptors (TLR). These receptors play a critical role in activating innate host defense mechanisms (Barreto et al., 2020, Bernardo et al., 2013). TLR are mostly expressed on phagocytic cells, but they are also found on MSC. It has been demonstrated that the stimulation with pro inflammatory cytokines such as INFγ and TNFα upregulate the expression of a subset of TLR, and therefore consequently increase the sensitivity of MSC in an inflammatory environment (Waterman et al., 2010, Mantovani et al., 2013, Raicevic et al., 2010). However, a prolonged stimulation with TLR-ligands results in downregulation of TLR subsets.(Mo et al., 2008)

This mechanism is one indication that the priming of MSC with cytokines can improve their immunoregulatory ability. Additionally, it has been demonstrated that stimulating MSC with INFγ and TNFα increases their immunosuppressive capacity by upregulating the expression of *ICAM-1* and *VCAM-1*, which are essential for lymphocyte–MSC adhesion (Ren et al., 2010). It appears that the inflammatory environment is crucial to form MSC to potent contributors.

1.3.1 Tumor Necrosis Factor alpha

TNFα is a glycoprotein with multifunctional effects in organisms. The cytokine is not detectable in heathy individuals, but elevated concentrations can be found in serum and tissue during inflammatory and infectious conditions. Physiological, it is crucial for an adequate immune response but can be harmful when over produced. TNFα is involved in the regulation of cell growth, metabolism, and differentiation of cell types. Due to activating the NF-kappa- light chain, the cytokine regulates the cell survival, proliferation, differentiation and migration (Robak et al., 1998, Bradley, 2008, Sarsenova et al., 2022).

1.3.2 Interferon gamma

INFγ is an important cytokine in the regulation of the immune system. Mostly produced by natural killer cells, the polypeptide activates macrophages, controls the differentiation of naive T cells to effector cells and supporting the production of proinflammatory cytokines. These examples show the major role INFγ plays in both the innate and the adaptive immunity. It has also the ability to inhibit viral replication directly and contributes in tumor control. However, an over or underproduction of INFγ is associated with many various diseases (Schoenborn et al., 2007, Boehm et al., 1997, Alspach et al., 2019). Additionally, INFγ increases the therapeutic potential of MSC by upregulating immunoregulatory genes in MSC (Vigo et al., 2017).

1.4 Extracellular vesicles

1.4.1 Definition

Extracellular vesicles are defined by the International Society for Extracellular Vesicles (ISEV) as particles released from cells, delimit by a lipid bilayer membrane and due to the absence of a nucleus, lacking the ability to replicate (Théry et al., 2018). They can originate either at the cell´s plasma membrane or within the cellular lumen (Johnstone et al., 1987, Harding et al., 1983). It has been demonstrated that all eukaryote cells release EV. These vesicles have been identified in multiple body fluids, including blood, urine, saliva, breast milk, ascites and semen (Colombo et al., 2014).

1.4.1.1 Subpopulations and their origin

EV are a heterogenous group with different origin and characteristics, and their nomenclature remains a topic of ongoing discussions. In this study, we adopt three commonly used terms. (Figure 2)

(1) Exosomes: These are formed intracellular through the inward blebbing of late endosomes. Late endosomes develop into multivesicular bodies (MVB), releasing exosomes through fusion with the cell membrane. Exosomes typically have a diameter ranging from 30 to 100nm (Harding et al., 1983, Johnstone et al., 1987). (2) Microsomes: These EV result from direct outward blebbing of the plasma membrane. With a diameter ranging from 100nm to 1µm, they are usually larger than exosomes. Except for tumour cells, the rate of released microsomes is lower than other EV but can be induced by cell activation and apoptosis (Cocucci et al., 2009, Colombo et al., 2014).

(3) Apoptotic bodies: Produced by blebs of apoptotic cells, these EV are widely accepted as the largest EV with diameters ranging from 1 to 5µm. However, there is evidence that fragments of dying cells can be even smaller than exosomes (Colombo et al., 2014, Mittelbrunn et al., 2012).

1.4.1.2 Term of Small EV

Due to the absence of definitive size restriction and contradictory definitions, a clear classification of EV based on their origin is challenging. Currently, achieving clarity may be only possible by observing the act of release by live imaging techniques. However, conducting such imaging is impractical or impossible in many experimental settings. Therefore, the ISEV suggests the use of the terms 'small EV' for those characterized by diameters smaller than 200 nm, and 'large EV' for those with diameters larger than 200 nm (Stein et al., 1991, Cocucci et al., 2009, Théry et al., 2018).

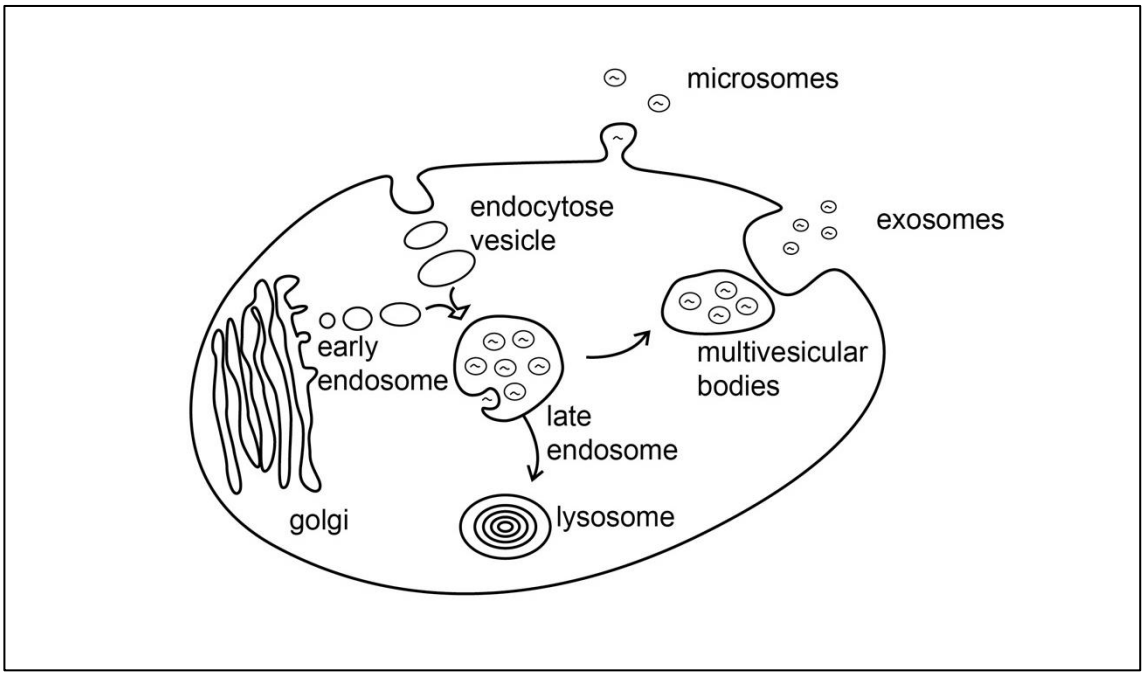

Figure 2: ***Origin of Exosomes and Microsomes***

Exosomes are formed intracellular through the inward blebbing of late endosomes. Late endosomes evolve through the fusion of early endosomes and endocytose vesicles. Late endosomes develop into multivesicular bodies, releasing exosomes through fusion with the cell membrane. Microsomes result from direct outward blebbing of the cell membrane. Adapted from (Harding et al., 1983, Johnstone et al., 1987, Théry, et al., 2018).

1.4.2 Components

The composition of EV is heterogenous as their original cells and due to their different subtypes of EV. Yet, there is evidence that some molecules are typical for EV. (Figure 3)

Proteins are the most researched molecules of the EV components. Due to their formation, endosomal proteins can be found in all exosomes, such as Alix, tumour susceptibility gene 101 (TSG101) and heat shock proteins (HSPs). Additionally, proteins of plasma membrane and cytosol are typical for exosomes as well as for other small EV (Bobrie et al., 2012, Cocucci et al., 2009, Simons et al., 2009). Prominent tetraspanins identified in EV include CD63, CD81, and CD9. They are often incorrectly labelled as “Exosome-markers”, because they were also detected in EV, surpassing the typical size range of exosomes. For example, CD9 has been found in larger EV by Bobrie *et al* (Bobrie et al., 2012) and suggesting a more widespread presence of CD9. Moreover, there is evidence indicating a large overlap off protein expression among subpopulations of EV (Kowal et al., 2016a). Nevertheless, it is generally understood that proteins originating from the nucleus, mitochondria, endoplasmic reticulum, and the Golgi complex are largely absent (Colombo et al., 2014). While studies on lipid components are limited, it has been demonstrated that phosphatidylserine, sphingomyelin, cholesterol, and ceramide are identified in both small EV and exosomes (Simons et al., 2009,Théry et al., 2009). A breakthrough in understanding EV was made in 2007 when it was discovered that exosomes carry nucleic acids, namely mRNA and miRNA (Valadi et al., 2007). The introduction of next generation sequencing revealed that various cell types exhibit distinct profiles of small RNA in exosomes. But it is suggested that the isolation method of exosomes can influence measurement and analysis of RNA content (Colombo et al., 2014).

Extracellular Vesicles	**Exosomes**	**Microsomes**	**Apoptotic bodies**
Released by	Multivesicular bodies	Plasma membrane blebbing	Blebs of apoptotic cells
Size	50- 100 nm	100-1000nm	> 1000nm
Origin	Endosomal	Cell membrane	Apoptotic cells
Content	Proteins, mRNA, miRNA, Lipids	Proteins, mRNA, miRNA, Lipids	Cell organelles, nuclear fractions,

	DNA, RNA, lipids, proteins,

Table 1: Overview of Extracellular Vesicles (Stein et al, 1991, Simons et al., 2009, Cocucci et al., 2009, Colombo et al., 2014)

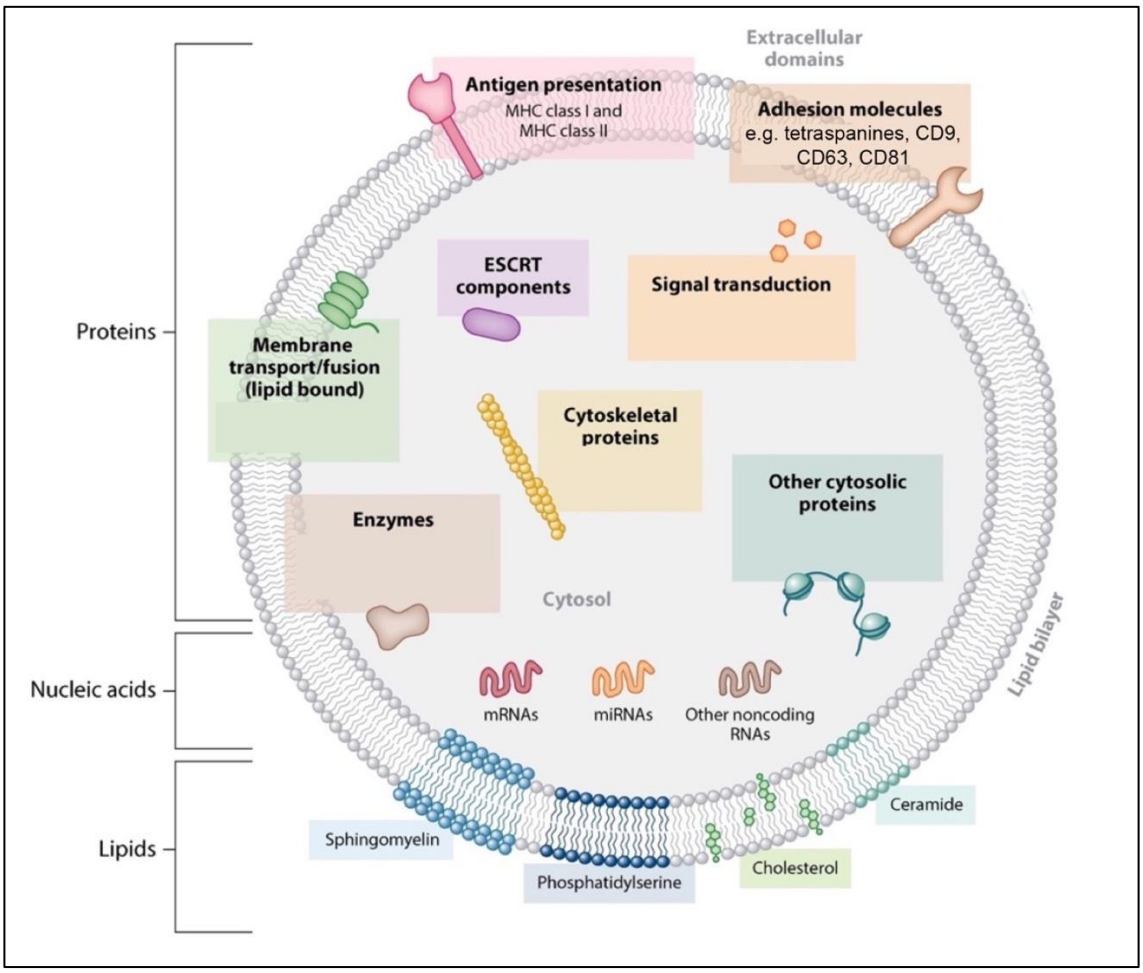

Figure 3: ***Components of Exosomes***

The three main components of Exosomes are: (1) Proteins, including the tetraspanins CD9, CD63 and CD81; (2) Nucleic acids, including mRNA, miRNA, and (3) Lipids in the bilipid membrane, including phosphatidylserine, sphingomyelin, cholesterol, and ceramide. Figure is adapted and modified by (Colombo et al., 2014).

1.4.3 Function

All human cells secrete EV. Generally, those vesicles mediate intercellular communication by transporting cargo molecules to recipient cells (Valadi et al., 2007). However, the specific function of EV is dependent on the cells which they originate from, suggesting a potentially extensive range of functions. Van der Pol reviewed over 500 studies and categorized the functions of extracellular vesicles into 6 as outlined in Table 2 (van der Pol et al., 2012). This suggests that vesicles

have the potential to be utilized for therapy, prognosis, and as biomarkers for health and disease.

Intercellular Signalling ○ Immune suppression ○ Antigen presentation ○ Inflammation ○ Tumour growth, metastasis, angiogenesis ○ Genetic information ○ Prions ○ Viruses
Antigen presentation
Cell adhesion
Waste management
Coagulation
Vascular function and integrity

Table 2: Function of Extracellular Vesicles (van der Pol et al., 2012)

1.4.4 Methods for Separation and Enrichment of small EV

According to the ISEV, complete isolation or absolute purification of EV is an unrealistic goal. Hence, they suggest using the term of “separation” or “enrichment”. Furthermore, they emphasize that the appropriate separation or enrichment method for an experimental set up has to be chosen individually.

For many years now, differential ultracentrifugation has been the primary method used, whereby the smallest vesicles (<100nm; including exosomes) are sedimented by ultracentrifugation at 100,000 × *g.* However, this established method poses several challenges. As mentioned earlier, the heterogeneity of the EV population lacks a strict size range. Consequently, separation only by size does not ensure purity. Additionally, ultracentrifugation can result in co-isolation and cross-contamination. It is crucial to acknowledge that high-speed centrifugation may lead to the presence of cell fragments and vesicle fusion (van der Pol et al., 2012, Théry et al., 2018).

Besides ultracentrifugation, there are many other methods with various protocols, each having its own advantages and disadvantages (Mateescu et al., 2017).

Another method of separation is using polyethylene glycol (PEG), which has been utilized for decades to concentrate and purify viruses (Albertsson & Frick, 1960). The hydrophilic polymer reduces solubility by lowering the hydration of EV and

leading to an aggregate, which can precipitate by low speed centrifugation (Weng et al., 2016, Rider et al., 2016a). Despite contrary opinions on the purity and yield of EV compared to other methods, it is commonly accepted that this method is suitable for high-volume concentration and downstream functional assays of EV (Lang et al., 2022a, Tian et al., 2020a, Jia et al., 2022a, Veerman et al., 2021b).

2. Objective

Given the increasing prevalence of OA there is a growing need for innovative strategies to address inflammation and joint damage. One extensively studied approach involves the use of mesenchymal stem cells. The significant role of MSC, including ASC, in inflammatory conditions is widely acknowledged. Moreover, there is supporting evidence that the presence of an inflamed environment enhances the immunomodulatory capabilities of MSC.

(1) In this study, we aimed to examine the impact of pro-inflammatory cytokines TNFα and IFNγ on ASC in vitro using qPCR, Annexin V/PI staining, and morphological analysis to assess and quantify their effects

The paracrine activity of ASC plays a pivotal role in maximizing their complete immunomodulatory potential. One component of the ASC secretome is extracellular vesicles.

(2) Another aim of this research was to separate EV from the conditioned medium. Achieving this required an optimization and review of cell culture conditions. Preliminary experiments were conducted to identify the most suitable method for EV separation. Subsequently, we established a modified approach utilizing polyethylene glycol.

(3) Protocols for NTA and Flow Cytometry were established to characterise EV and identify differences compared to a control.

(4) Returning to the therapeutic approach for OA, our focus was to investigate the impact extracellular vesicles (EV) have on chondrocytes in vitro.

In summary, the research questions of this study were:

1) **What is the impact of cytokine priming on ASC?**
2) **How can a suitable EV separation/concentration method be established?**
3) **What distinct characteristics are exhibited by EV derived from cytokine-primed ASC?**
4) **Do EV from cytokine-primed ASC induce different effects on chondrocytes compared to EV from non-primed cells?**

3. Material & Methods

3.1 Ethical Aspects

Human lipoaspirates were obtained through liposuction by a surgeon according to actual surgical standards, after written informed consent signed by the patients as approved by the ethics committee of Ludwig-Maximilians-University, Munich (275-16). Medical indicated total knee arthroplasty was performed by a surgeon according to actual surgical standards, after written informed consent signed by the patients as approved by the ethics committee of Ludwig-Maximilians-University, Munich (19-177). All patients have been tested negative for HIV (human immunodeficiency virus), HCV (hepatitis C virus) and HBV (hepatitis B virus).

3.2 Cell Culture of SCP1 Cells

To develop the best suited experimental set up immortalized stem cells (SCP1, kindly provided by W. Böcker, LMU (Böcker, et al., 2008) were used. Cells were seeded in a density between $1x10^6$ and $2x10^6$ per T175 flask. Cells were cultured in complete DMEM medium, consisting of DMEM (Thermo Fisher Scientific, Waltham, Massachusetts, USA), 10% FBS (Sigma-Aldrich, St. Louis, Missouri, USA) and 100 U/ml penicillin and 100 µg/ml streptomycin (Life Technologies, Carlsbad, California, USA). When cells reached a confluence of 80-90% they were washed three times with 1x phosphate buffered saline (PBS; Sigma-Aldrich) before medium was changed to serum free medium or 10% exo-depleted FBS (Ref.: A27208-01, Gibcon ™, Thermo Fisher Scientific) consisting medium. Cell culture was produced under normal culture conditions in a Water Jacketed CO2 Incubator TC 230 (Thermo Fisher Scientific) under constant conditions of 37°C and 5% CO_2

3.3 Adipose Derived Stem Cells

3.3.1 Isolation and Characterisation of ASC

Human adipose derived stem cells isolated from white adipose fat tissue were used for experiments. Cells were obtained from abdominal lipoaspirates from 5 female donors (average age 61,6 years) recruited at the Clinic for General, Trauma and Reconstructive Surgery of the Ludwig-Maximilians-University (LMU, Munich, Germany). All lipoaspirates were harvested through liposuction, performed with the LipiVage system (Bondimed, Ohlsdorf, Austria) by a surgeon ac-

cording to common surgical standards. All patients were in general healthy condition. The cells were isolated, characterized and kindly provided by S. Taha, LMU (Taha et al., 2020). In brief, ASC were isolated with a semi-automated centrifuge system followed by the manufacturer's protocol using their enzyme blend and lactated Ringer's solution (Fresenius Kabi, Bad Homburg, Germany). Stem cell properties were proven by differentiating a proportion of isolated cells into osteogenic, adipogenic and chondrogenic lineage.(Domenis et al., 2015)

3.3.2 Cryopreservation of ASC

For long term storage ASC were put into a liquid nitrogen tank. Therefore, after the last centrifugation step the cell pellet was resuspended in ice cold freezing medium consisting of DMEM (Thermo Fisher Scientific), 20% FBS (Sigma-Aldrich) and 1% Dimethylsulfoxid (DMSO, AppliChem, Darmstadt, Germany) (Table 3). Aliquots were prepared and stored on dry ice until frozen completely. Subsequently, the cryovials (Nalgene by Thermo Fisher Scientific) were placed in a -80°C freezer for maximum one week, before the vials were transferred into liquid nitrogen for long-term storage.

3.3.3 Thawing of ASC

To thaw cells, cryovials were placed in a water bath at 37°C until the suspension melted. Afterwards, cells were mixed with fresh DMEM media (Thermo Fisher Scientific) in a 15 ml falcon tube and centrifuged for 5 min at 500g. Next, the supernatant was aspirated and the cells were resuspended in fresh and pre-warmed DMEM supplemented with 10% FBS (Sigma-Aldrich), 100 U/ml penicillin, 100 µg/ml streptomycin (Life Technologies) and 10ng/ml human *FGF2*-growth factor (Miltenyi Biotec, Bergisch Gladbach, Germany) (Table 4). Finally, the cells were transferred into culture flasks T-175 (Thermo Fisher Scientific). Complete growth DMEM media was changed after 24h in order to remove non-attached and dead cells.

Freezing Medium
79% Dulbecco's modified Eagle's medium (DMEM)-high glucose
20% fetal bovine serum (FBS)
1% Dimethylsulfoxid (DMSO)

Table 3: Freezing Medium

Growth Medium
89% DMEM high glucose
10% FBS
1% Penicillin + Streptomycin
10ng/ml FGF-2-growth factor

Table 4: Growth Medium

3.3.4 Cell Culture of ASC

For general expansion and subsequent experiments, cells were handled under sterile conditions in a cell culture laminar flow hood (Heraeus Instruments, Germany) at all time. After thawing of ASC, Passage (P) 1 cells were cultured with a seeding density of 2 x10 ^{6} per T175 flask (Thermo Fisher Scientific). Complete Growth Medium (Table 4)was used. At a confluence of 80-90% cells were detached with Trypsin/EDTA (Sigma-Aldrich) as described below and cultured in new T175 flasks with again a seeding density of 2 x10 ^{6} per flask. When P2 cells reached a confluence of 90% they were divided into a control group and primed group. The control group was formed with cells cultured in Standard Medium (Table 5)consisting DMEM (Thermo Fisher Scientific) supplemented with 10% FBS (Sigma-Aldrich), 100 U/ml penicillin and 100 µg/ml streptomycin (Life Technologies). The cells in the primed group were cultured in standard culture medium supplemented with 25ng/ml *TNFα* (R&D Systems, Minneapolis, Minnesota, USA) and 25ng/ml *INFγ* (Perotech Sciences, Markham, Canada) (Priming Medium;Table 6) for proinflammatory priming. After 48h, both groups were washed three times with PBS (Sigma-Aldrich) and cultured for another 48h in serum free medium (Table 7). All cell culture was produced under normal culture conditions in a Water Jacketed CO_2 Incubator TC 230 (Thermo Fisher Scientific) under constant conditions of 37°C and 5% CO_2.

Standard Medium (Control Group)
89% DMEM high glucose
10% FBS
1% Penicillin + Streptomycin

Table 5: Standard Medium (Control Group)

Priming Medium (Primed Group)
89% DMEM high glucose
10% FBS
1% Penicillin + Streptomycin
25ng/ml TNFα
25ng/ml INFγ

Table 6: Priming Medium (Primed Group)

Serum free Medium for EV harvest
99% DMEM high glucose
1% Penicillin + Streptomycin

Table 7: Serum free Medium

3.3.5 Flow Cytometry Assay for Cell Apoptosis Detection and Cell Viability

Apoptosis of the ASC was analysed with the Annexin V-APC /PI detection Kit (Cat.No.: 88-8007-72; Thermo Fisher Scientific) with a FACScalibur (Becton Dickinson, Heidelberg, Germany) following the manufacturer's protocol. Therefore, after collecting the conditioned medium, cells were washed with PBS (Sigma-Aldrich) and 5ml Accutase™ (StemPro™ Accutase™, Thermo Fisher Scientific) was added to each T175 flask to detach cells from surface. After 5 minutes incubation at 37°C a double volume of complete standard DMEM media was added to the flask to neutralise the effect of the Accutase™ and the suspension was put in a falcon. The cells were centrifuged at 300xg for 5 minutes and the cell pellet was resuspended in PBS (Sigma-Aldrich). After another centrifugation step of 500xg for 5 minutes the cell pellet was resuspended in the 1x binding buffer of the Kit and counted with the Neubauer chamber method. $1x10^6$ cells were added to 1ml of binding buffer. Four samples with each 10.000 cells were prepared for unstained, single labelling or double labelling testing. Annexin V samples were incubated 10 minutes in the dark at room temperature, followed by 500xg centrifugation for 5 minutes. The cell pellet was then washed with the supplied binding buffer and resuspended in the needed amount of binding buffer. (Table 8) For the propidium iodide (PI) staining 5µl of PI was added. In the end each sample had a total volume of 200µl.

	unstained	AnnexinV Single labelling	PI single labelling	Double labelling
Cell suspension (100 cells/µ)l	100µl	100µl	100µl	100µl
Annexin V	-	5µl	-	5µl
Binding Buffer	100µl	200µl	95µl	195µl
PI	-	-	5µl	5µl

Table 8: Annexin V - PI labelling protocol

The samples were visualised with FACScalibur (Becton Dickinson). Flow Cytometry data was analysed with FlowJo software (Tree Star, Ashland, Oregon, USA).

3.4 Passaging of Cells and Determination of Cell Number

Cells were washed with 1x PBS (Sigma-Aldrich) and then detached by covering with prewarmed 1x Trypsin/EDTA (Sigma-Aldrich) for 5 min at 37°C. A double volume of standard DMEM media (Table 5) was added to neutralize the effect of trypsin. The cell suspension was transferred in to a 15ml falcon and the flask was washed again with DMEM media, which was added to the 15ml falcon as well to collect all cells. Afterwards cells were centrifuged at 500g for 5 minutes. The supernatant was removed and cells were resuspended thoroughly with standard DMEM. To determine the total number of cells per milliliter, 10µl cell suspension was injected into a Neubauer chamber (Brand, Grafrath, Germany), cells were counted in all four quadrants (A, B, C and D) divided by 4 and multiplied by 10^4: cells/ml = ((A+B+C+D)/4) *10^4.

3.5 Protein Extraction of Cells

For protein extraction a mixture of 1 tablet protease inhibitor (complete Mini EDTA-free Protease Inhibitor, Roche, Penzberg, Germany) and 10 ml of ice cold radioimmunoprecipitation assay (RIPA; Table 9: RIPA Buffer) buffer was prepared. After collecting the media, the cells were washed with PBS (Sigma-Aldrich) before 3 ml of the RIPA-mixture was added to a T175- flask. To detach all cells, a cell scraper was used carefully. The solution was transferred to an Eppendorf tube and put on ice for 20 minutes. After a centrifugation step with

10000xg at 4°C for 15 minutes the supernatant was collected and stored at -80°C until further use.

Radioimmunoprecipitation assay (RIPA) buffer	
0.1% Sodium dodecylsulfate (SDS)	150 mM NaCl
1% Sodium deoxycholate (NaDOC)	10 mM (EDTA) Ethylenediaminetetraaceticacid
1% Triton X-100	20 mM NaF
50 mM Tris pH 8.2	Filtered distilled water

Table 9: RIPA Buffer

3.6 Separation of Extracellular Vesicles with PEG

Serum free medium was pooled from each group. 150ml of each group of every donor was centrifuged two times (300xg 10 minutes, 3000xg 30 minutes) to remove cell debris and dead cells. The supernatant was filtered through a 0,45µm CA-Membrane (Kuhnle, Karlsruhe, Germany) to remove larger microvesicles. One filter was used for 15-20 ml medium. Cleaned medium was concentrated with a 100k Amicon Filter-System (Millipore, Carrigwohill, County Cork, Ireland) with the concentration factor of 75. 15ml was added to one Amicon Filter System and centrifuged at 4000xg for 15 minutes. Each filter System was used three times. The concentrated medium was pooled from each donor and each group and 1:5 Polyethlyenglycol solution (500mg PEG 8000 (Promega, Fitchburg, Madison, USA) /ml PBS;Table 11) was added to the medium and incubated at 4°C overnight with slow rotation. On the next day, samples were centrifuged (1500xg 30´) and the pellet was resuspended in 100µl PBS (Sigma-Aldrich) + 25mM Trehalose (Sigma-Aldrich) to avoid adhesion of EV (Bosch et al., 2016).(Figure 4)

Material for EV enrichment	
0,45µm CA-Membrane	Kuhnle, Germany
100k Amicon Filter- System	Millipore, Ireland

Table 10 Material EV- Enrichment

Polyethlyenglycol (PEG) - solution	
5g PEG 8000	Promega, USA
10ml filtered PBS	Sigma-Aldrich, USA

Table 11: PEG-solution

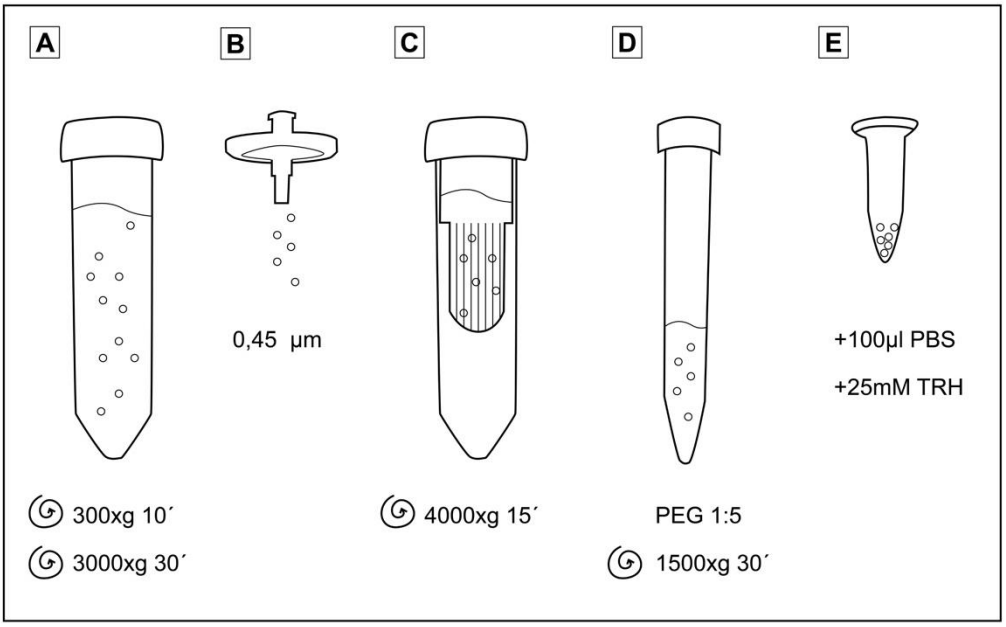

*Figure 4: **EV separation steps***

(A) Medium was centrifuged at 300xg for 10`, the supernatant was centrifuged a second time at 3000xg for 30`(B) Supernatant was cleaned with a 0,45µm CA-Membrane filter (C) Medium was transferred to a 100k Amicon Filter-System and centrifuged at 4000xg 15` (D) Concentrated medium was mixed with 1:5 PEG solution overnight, and EV were spun down at 1500xg for 30`(E) PEG-EV pellet was resuspend in PBS and TRH and transferred in a fresh tube

3.7 Protein Extraction of EV

To extract the proteins of the EV a mixture of 1 tablet protease inhibitor (complete Mini EDTA-free Protease Inhibitor, Roche) and 10 ml of ice cold radioimmunoprecipitation assay (RIPA) buffer (Table 9) was prepared. Samples of 50µl of EV in PBS and 50µl of RIPA buffer were put on ice for 20 minutes. After a centrifugation step at 10000xg at 4°C for 5 minutes, the supernatant was collected and directly used for protein measurement.

3.8 EV-Characterisation

3.8.1 Micro BCA-Assay

To detect protein concentrations of cell lysates and isolated EV a Micro BCA™ Protein Assay Kit (Cat.No.23235, Thermo Fisher Scientific) was used following the manufactures protocol. The protein concentration was determined based on a chemical reaction where BCA interact with the cuprous cations that are reduced by the proteins in an alkaline media. As a result, an intense purple-colored reaction is observed and the intensity of the color correlates with the protein concentration. In brief, a standard curve up to 200µg/ml was made with diluted albumin provide by the kit. Samples of EV and cell lysates were diluted with distilled water. A RIPA buffer dilution of 1:10 was guaranteed at all time. A 96 well micro plate was used. In each well 150µl of the sample or the standard and 150µl of the provided working reagent was added and put on a plate shaker for 30seconds. After 2 hours of incubation at 37°C the absorbance was measured at 562nm on a plate reader (Photometer Multiscan FC; Thermo Fisher Scientific). All samples were prepared in duplicates

3.8.2 Western Blot

3.8.2.1 SDS-Polyacrylamide gel electrophoresis (PAGE)

For the gel electrophoresis, polyacrylamide gels were prepared consisting ¾ 10% APS resolving gel and ¼ 5% APS stacking gel with a 0,75mm thickness. Protein extracts were mixed with 4x Laemmli buffer and filled up with distilled water to the need amount. Depending on the later used antibody, reducing or non-reducing Laemmli buffer was applied. Gels were loaded with 5µl protein and a standard protein ladder (peqGOLD Protein Marker IV, VWR GmbH, Darmstadt, Germany). The electrophoresis was performed in 1x Running buffer and at 60mA for 1 hour for one gel.

Instruments	
Mini-ProteanTetra®	BioRad, Hercules, CA, USA

Gels
30% Acrylamide
1M Tris
(pH 8,8, resolving gel; pH 6,8 stacking gel)
10% SDS
10% APS
TEMED

1x Running Buffer
25mM Tris pH 8,3
0,1% SDS
192mM Glycin

4x Laemmli Buffer + ß-mercaptoethanol
50mM Tris-base pH6,8
10%Glycerol
2,5%SDS
7,5% ß-mercaptoethanol
0,005% Bromphenolblau

Table 12: SDS-Polyacrylamide gel electrophoresis

3.8.2.2 Protein Transfer

The protein transfer was performed by the vertical wet transfer method. The polyacrylamide gel loaded with proteins, filter paper and fiber pads were equilibrated for 15 min in 1x blotting buffer. The polyvinylidene fluoride (PVDF, Roche) membrane was activated by soaking into methanol for few seconds, rinsed in water and placed in 1x blotting buffer. Subsequently, the cassette was assembled and the protein transfer was performed in a Mini Trans-blot® cell at 4°C with a constant voltage of 30 V overnight.

Instruments	
Mini Trans-blot® cell	BioRad, Hercules, CA, USA

Table 13: Instruments

1x Blotting Buffer
2,5mM Tris base (pH8,3)
192mM Glycin
10%Methanol

Table 14: Blotting Buffer

3.8.2.3 Protein Immunodetection

For immunodetection the PVDF-membrane was washed with a TBS-Tween20 wash buffer and then blocked with a 5% skim milk solution for 90 minutes. Afterwards the membrane was washed again, followed by incubation with the primary antibody in appropriate dilution at 4°C overnight. The next day the membrane was washed carefully and incubated with anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibody (Rockland, Pottstown, Pennsylvania, USA) 1:2000 diluted in the blocking solution at RT for 1 hour. Finally, the membrane was washed 3 times for each 10 minutes with washing buffer. Proteins on PDVF membrane were visualized by using chemiluminescent Immobilion Western HRP-Substrate (Merck) and the luminescent image analyzer Image Quant LAS 4000 mini (GE Healthcare, Chicago, Illinois USA).

Wash Buffer
10mM Tris base (pH 7,4)
150mM NaCl
0,05% Tween 20

Table 15: Wash Buffer

Blocking Solution
5% skim milk powder
Wash buffer

Table 16: Blocking solution

Primary antibodies				
	Manufacture	**Cat. No.**	**Host**	**Dilution**
CD81	Millipore Carrigwohill, County Cork, Ireland	MABF2061	Mouse	1:1000
CD63	BDSciences, Franklin Lakes, New Jersey, USA	556019	mouse	1:2000
GP96	R&D System Minneapolis, Minnesota, USA	MAB7606	Mouse	1:1000

Table 17: Primary antibodies Western Blot and EV bead flow cytometry

3.8.3 Nano Tracking Analysis

Nano Tracking Analysis was performed in cooperation with Prof. Michael Pfaffl at Technische Universität München, Weihenstephan. Particle concentration and size distribution within the sample were determined by ZetaView® Nanoparticle Tracking Analyzer PMX 110 (Particle Metrix GmbH, Inning am Ammersee, Germany). EV samples were diluted in PBS (Sigma-Aldrich) to a final volume of 1ml. For each measurement, two measurement cycles were performed by scanning 11 positions each and acquiring 60 frames per second under the following settings: pre-acquisition parameters were set to a sensitivity of 80; Shutter was set to 70. Cell temperature was set at 25°C and trace length to 15. (Bachurski et al., 2019) After recording, the videos were analysed with the built-in ZetaView software 8.05.11 SP1 (Particle Metrix GmbH) with specific analysis parameters: minimum particle brightness: 20; minimum size of 5 pixels, maximum size of 1000 pixels and PSD nm/class of 10 and PSD classes/decade of 10.

3.8.4 EV Bead Flow Cytometry

Flow Cytometry was performed in cooperation with Prof. Michael Pfaffl at Technische Universität München, Weihenstephan. For each sample $5x10^7$ particles/EV were used and $4{,}5x10^9$ SiMAG-Carboxyl magnetic beads with 1µm diameter (Chemicell GmbH, Berlin, Germany) were added to each EV sample and filled up with filtered PBS (Sigma-Aldrich) to a final volume of 1ml. Samples were incubated 2 hours at 25°C to enable nonspecific binding of EV and proteins to

the aldehyde covered surface of the beads. Followed by 30 minutes incubation with EV-free FBS (EF-FBS, Sigma-Aldrich) to saturated unbound areas of the bead surface. The EV-bead complexes were further stained with the same master mix of CD9, CD63 and CD81 antibodies (2.5 µl/ml in 200 µl, Table 17) for 1 hour at 37°C. After each incubation the samples were washed. Therefore, the tube with the sample was placed on a magnetic separator for 60 seconds. The supernatant was removed while the tube remained on the separator. 1 ml of PBS + 0,1% EF-FBS was added and the sample was washed by gently pipetting up and down. This step was repeated two times. The EV-bead complexes stained with antibodies were visualized with BD LSR Fortessa™ (BD Sciences). A negative control was obtained by coupling the beads to a plain DMEM medium. Flow Cytometry data were analysed by FlowJo software (Tree Star).

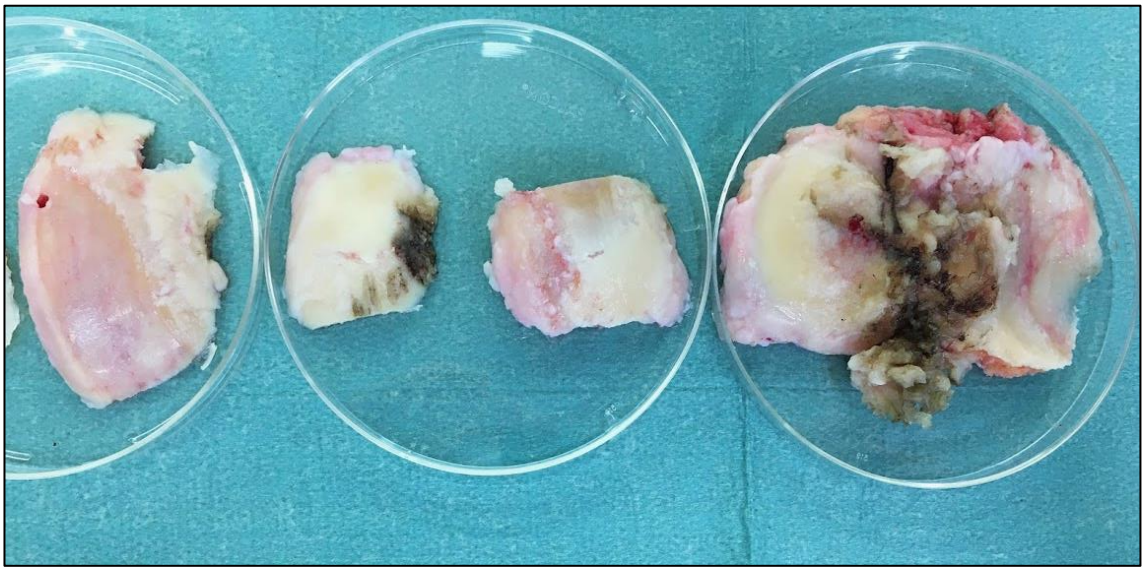

Figure 5: ***Initial knee tibia plateau as source of chondrocytes***

3.9 Chondrocytes: Culture Conditions and EV Treatment

Chondrocytes were isolated from a 73-year-old male donor undergoing total knee arthroplasty. The whole tissue explant was stored in PBS (Sigma-Aldrich) at 4°C until the next day. The explant was washed several times with PBS before areas of the tibia plateau were cut. Tissue cuts were left in the digestion solution over night at 37°C with nutation. Digestion solution consisted of DMEM F12 +15mM HEPES (Life Technologies) supplemented with 150 U/ml penicillin and 150 µg/ml streptomycin (Life Technologies), 1% FBS (Sigma-Aldrich) and 2mg/ml Collagenase II (Worthington, Lakewood, New Jersey, USA) and was sterile filtered (Table 18). On the following day, the medium was filtered through a 70µm nylon cell strainer (Falcon, UK). Cells were centrifuged two times at 500xg for 5 minutes

before seeded in a density of 3,5 x10 ^{6} per T175 flask. Culture Medium included DMEM F12 (Bio&Sell, Feucht, Germany),10% FBS (Sigma-Aldrich) and 150 U/ml penicillin and 150 µg/ml streptomycin (Life Technologies) (Table 19). Finally, chondrocytes were seeded in a density of 80000 cells/well on a 24 well plate. At a confluence of 90%, the medium was changed to serum free condition, consisting of culture medium supplemented with 1% insulin, transferrin and natriumselenit solution (ITS +3; Sigma-Aldrich) instead of FBS to synchronise the cells (Table 20). After 15 hours, fresh medium was applied and cells were treated with 10ng/ml IL1ß (Milteny Biotec) and 50x10^{6} particles/ml of the EV from either the control or primed group (Table 21). Controls were only treated with 10 ng/ml IL1ß. Cells were treated for 24 hours. All cell culture was produced under normal culture conditions (37 °C, 5% CO_2, humidified).

Digestion solution
DMEM F12
15mM HEPES
1% FBS
150 U/ml penicillin
150 µg/ml streptomycin
2mg/ml Collagenase II

Table 18: Digestion solution

Standard medium Chondrocytes
89% DMEM F12
10% FBS
1% Penicillin + Streptomycin

Table 19: Standard medium Chondrocytes

Serum Free medium Chondrocytes
98% DMEM F12
1% ITS+3
1% Penicillin + Streptomycin

Table 20: Serum Free medium Chondrocytes

Treatment medium Chondrocytes (24h)
98% DMEM F12
1% ITS+3
1% Penicillin + Streptomycin
10ng/ml IL1ß
$5x10^7$ particles/ml Measured by NanoTracking

Table 21: Treatment medium Chondrocytes

3.10 RNA-Isolation of Treated Chondrocytes and ASC

After collecting conditioned medium, 200µl of RLT-lysis-Buffer (Qiagen, Venlo, Netherlands) + 1% ß-mercaptoethanol was added to each well of chondrocytes to isolate the RNA and stored at -80°C until further use. For the ASC, the flasks were washed with PBS (Sigma-Aldrich) and 3ml of the RLT-lysis buffer + 0,1% ß-mercaptoethanol was added to lyse the cells. After 10 minutes incubation at room temperature cells were detached using a cell scraper (Sarstedt, Nümbrecht, Germany). For homogenization of the lysates the QIAshred spin columns were used (Qiagen). Then, the lysate was transferred to a column and centrifuged for 2 minutes at 10000xg. The flowthrough was used for further steps. RNA was isolated following the manufacturer's protocol of the RNeasy Kit (Qiagen). To purify the isolated RNA, 70% ethanol in 1:1 ratio was added to the lysate and afterwards transferred into a RNeasy® Mini spin column and centrifuged for 15 sec at 10000rpm. RW1-Buffer was added and again centrifuged for 15sec at 10000rpm. On-column DNase digestion was performed with 10µl DNAse (Qiagen) solved in 70 µl RDD-Buffer for 15 min. Another washing step with RW1-buffer and subsequent centrifugation followed. Next RPE-buffer was added and centrifuged for 2 min at 10000rpm. This step was repeated once and the columns were transferred into new tubes to be dried by a further centrifugation step for 1min at 10000rpm. To elute total RNA, RNase free water was added to columns and centrifuged. To evaluate concentration and purity of the RNA, measurements with a spectrophotometer (NanoDrop, Thermo Fischer Scientific) at A_{260} and A_{280} were performed.

3.11 cDNA preparation

cDNA was synthesised out of 100ng RNA using the cDNA Synthesis Kit (Biozym, Oldendorf, Germany) following the manufacturer's protocol. RNA samples corresponding to 1 µg RNA were filled up to 11 µl with H_2O, afterwards 1 µl hexamer-random primers (25 µM) and 0,5µl Oligo (dT)- primer (10 µM) were added and

samples were incubated for 10 min at 65°C for denaturation. For the next step a mixture of 2µl dNTP (10 mM), 0,5µl RNase-inhibitor (40 U/µl), 4µl cDNA-synthesis buffer and 1µl Reverse Transcriptase (10 U/µl) was prepared and added to each sample. The mixtures were incubated in a thermocycler (PEQLAB Biotechnologie GmbH, Germany) for 1 hour at 50°C following the cDNA synthesis program (Table 22).

RNA denaturation mix	
RNA	1µg
Hexamer random primers (25µM)	1µl
Oligo (dT)- primer (10 µM)	0,5µl
cDNA-synthesis mix	
dNTP	2µl (10 mM)
RNase-inhibitor	0,5µl (40 U/µl)
cDNA-Synthesis Buffer	4µl
Reverse Transcriptase	1µl (10 U/µl)
program for cDNA-synthesis	
25°C 10’	
50°C 60’	
85°C 5’	
4°C forever	

Table 22: cDNA preparation

3.12 Quantitative Reverse Transcriptase-PCR (qRT-PCR)

Quantitative Reverse Transcriptase-PCR (qRT-PCR) was performed using the Taq Man Probes for BCL2, hCCND1, hTNFα, hCOX-2, hIL6, hMMP-13, Peptidyl isomerase A (PPIA) (all primers were from Integrated DNATechnologies, Coralville, Iowa, USA). PCR reaction was carried out in a LightCycler® 96 instrument provided with LightCycler® 96 software (Roche, Penzberg, Germany). The relative gene expression was normalised in relation to the housekeeping gene PPIA by the comparative ΔΔCt (crossing point) method. cDNA samples, dH_2O and Prime Time Gene Expression Master Mix were pipetted in duplicates in a 96-well plate (Table 24). A short centrifugation (30 sec) followed to collect samples on the bottom of each well. The n-fold change of gene expression levels relative to

PPIA was performed by comparative 2-ΔΔCt method. All samples were prepared in duplicates.

Samples Reaction Recipe	
Prime Time Gene Expression Master Mix	10µl
cDNA (diluted 1:5 in dH_2O)	5µl
Primer Mix	1µl
dH_2O	0,5µl

Table 23: Samples Reaction Recipe

Primers from IDT	**Cat. No.**
hBCL2	Hs.PT.56a654557
hCCND1	Hs.PT56a4930170
hIL6	Hs.PT.5840226675
hMMP13	Hs.PT.58.40735012
hPPIA	Hs041944521_s1
hPTGS2 (COX2)	Hs.PT.5877266
hTNF	Hs.PT58.45380900

Table 24: Primers

3.13 Enzyme-Linked Immunosorbent Assay (ELISA)

To detect and quantify the response of the EV treatment in the cell culture supernatant of the chondrocytes, several enzyme-linked immunosorbent assays were performed. Targeting proinflammatory factors, TNFα, IL6 and COX2 were chosen. To show cell viability BCL2 plus LDHB were used as markers. MMP13 was selected as a marker for cartilage degeneration. All experiments followed the manufactures protocol of the corresponding kit (Table 25). In brief, samples were diluted with sample diluent of each kit. First, 100µl of diluted samples and standards were pipetted into provided or prepared wells, whereas negative controls were loaded with 100µl of PBS (Sigma-Aldrich). Next, samples were covered and placed in an incubator (Memmert, Germany) for 1h - 2h at room temperature. After washing the wells, the corresponding antibody mixture was added, followed by another incubation step. Next, samples were washed again and the colour reagent was added to each well and the plates were incubated again. Another

washing step was performed. Then, the reaction was stopped with supplied stop solution, the optical density was measured with the multiskan FC microtiter-plate reader (Thermo Fischer Scientific) at 450nm. Concentrations were calculated against a standard curve.

Target	Manufacture	Cat.Nr.	Sample dilution
TNF-α	R&D Systems	DTA00D	1:10
IL6	R&D Systems	D6050	1:100
COX2	abcam	ab267646	1:10
BCL2	R&D Systems	DYC827B-2	1:10
LDHB	abcam	ab183367	1:2
MMP13	R&D Systems	DM1300	1:100

Table 25: ELISA Kits

3.14 Statistics

Quantitative data were analyzed and visualized with GraphPad Prism 5 software (GraphPad, La Jolla, CA). Bar charts show median values and standard deviation. For comparison of two groups, paired or unpaired t-tests or Mann-Whitney-U-tests were performed. For comparison of more than two groups ANOVA was used. A P-value of 0,05 was taken as statistically significant. Every test was performed in duplicates.

3.15 Table of Common Chemicals

Sodium dodecylsulfate (SDS)	Sigma-Aldrich,USA
Sodium deoxycholate (NaDOC)	Sigma-Aldrich,USA
Triton X-100	Thermo Fisher Scientific, USA
NaCl	Thermo Fisher Scientific, USA
Ethylenediaminetetraaceticacid (EDTA)	Sigma-Aldrich,USA
Natriumfluorid	Sigma-Aldrich,USA
Tris base	Merck, Germany
Polyethlyenglycol PEG 8000	Promega, USA
Acrylamide	Sigma Aldrich, USA
Amoniumperocidsulfat (APS)	Sigma Aldrich, USA
Tetraacetylethylenediamine (TEMED)	Sigma Aldrich, USA
Glycin	Sigma Aldrich, USA
Methanol	Merck, Germany
Skim milk powder	Sigma Aldrich, USA
ß-mercaptoethanol	Sigma Aldrich, USA
Bromphenolblau	Merck, Germany

4. Results

4.1 Preliminary Experiment

Developing the setup for this study, preliminary experiments were employed. It was shown that with the PEG-Method typical EV-related markers such as CD63 and CD81 could be detected in the Western Blot (Figure 6), whilst a negative marker (Kowal et al., 2016b) as GP96 was not present (Figure 7).

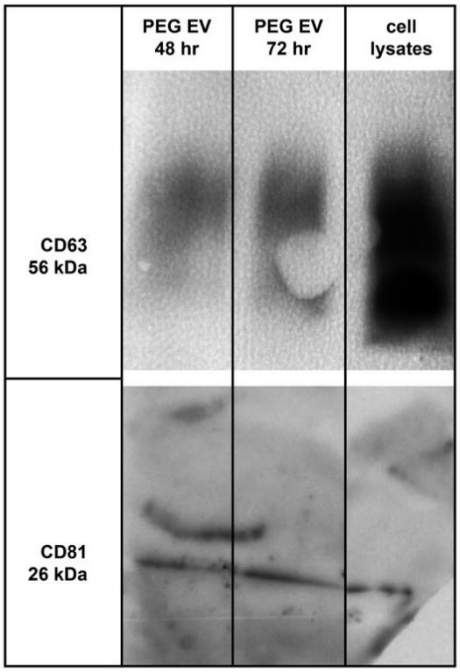

Figure 6: ***CD 63 + CD 81 Western Blot***

CD 63 and CD 81expressions were detected by western blotting. EV were isolated by the PEG method after 48 hours compared to 72 hours. Cell lysates were used as a positive control.

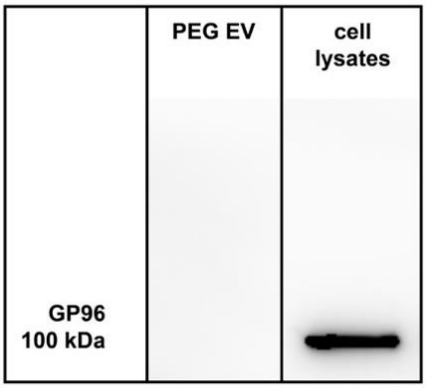

Figure 7: ***GP96 Western Blot***

EV isolated with the PEG method showed no expression of GP96. Cell lysates expressed GP96 and were used as a positive control

To optimize the culture conditions, medium with commercial Exosome depleted FBS (#A2720801 Gibcon ™, Thermo Fisher Scientific) was compared to medium without any serum. Under identical cell culture conditions, the conditioned medium supplemented with commercial exosome-depleted FBS produced a large pellet after the final PEG centrifugation step. In contrast, the conditioned medium without serum resulted in a significantly smaller pellet. Although the commercial exosome-depleted FBS did not show a positive signal in the Western blot, potentially indicating its purity, we concluded that the difference in pellet size is likely due to co-isolation or impurity (Figure 8).

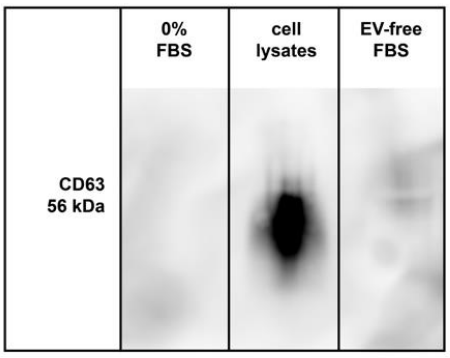

Figure 8: ***CD 63 expression in serum free medium compared to Exosome depleted FBS medium (EV-free FBS)***

No CD63 expression was detected in EV from Exosome depleted FBS culture conditions. No CD 63 expression detected from the same volume of serum free culture conditions. Cell lysates were used as a positive control.

To obtain more final EV-material the volume of medium was scaled up to 150ml. The protein concentration of EV was measured with a micro BCA-Assay after 48h and 72h and showed a higher outcome after 48 hours (Figure 9). These findings suggest a higher EV production after 48hours.

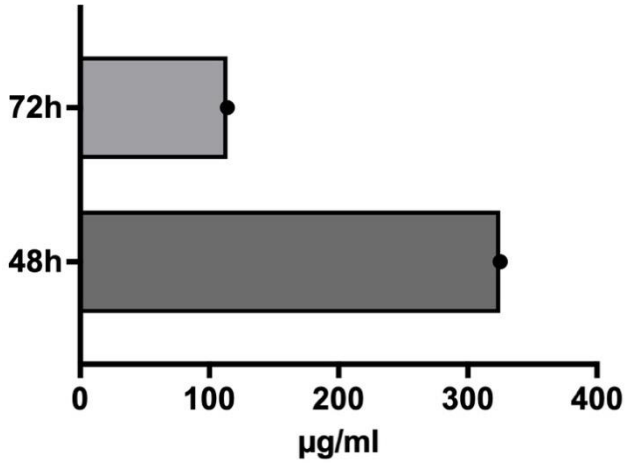

Figure 9: ***Protein concentration of EV***

Comparing protein concentration based on duration of serum free culture condition of ASC with BCA-Assay. After 48 hours a concentration of 325µg/ml was measured and after 72 hours 114µg/ml.

4.2 Primed ASC

4.2.1 Quantitative PCR : Impact on gene expression

ASCs were primed with pro-inflammatory markers TNFα and INFγ for 48h in order to mimic an inflammatory environment. To observe the effect of the cytokines on the ASC gene expression level, a qPCR was performed on cell samples and compared to their controls. Figure 10 indicates the increase of *TNFα*, *COX2*, *IL6* and *NOS*, which points out an inflammatory response of the priming. *NOS* and *TNFα* reached a level of significance ($p > 0.05$).

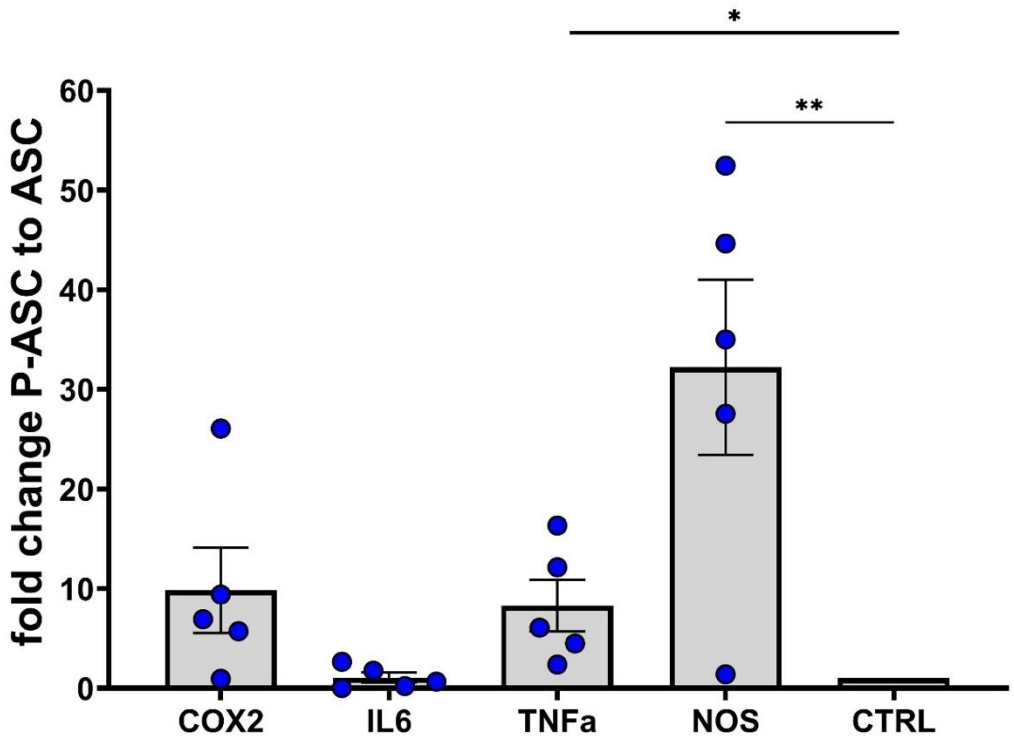

Figure 10: **Proinflammatory effect of TNFα and INFγ**

*Primed ASC compared to ASC. A qPCR (n=5) was performed and detected an upregulation of NOS, COX2, IL6 and TNFα. NOS reached a level of significance (**p value: 0,008), as well as TNFα (*p value: 0.025)*

Looking at the gene expression of *BCL2* as an apoptotic regulator there was nearly no change due priming the cells. On the other hand, *CCND1* as a proliferation marker, showed a statistically relevant up regulation through the priming process (Figure 11).

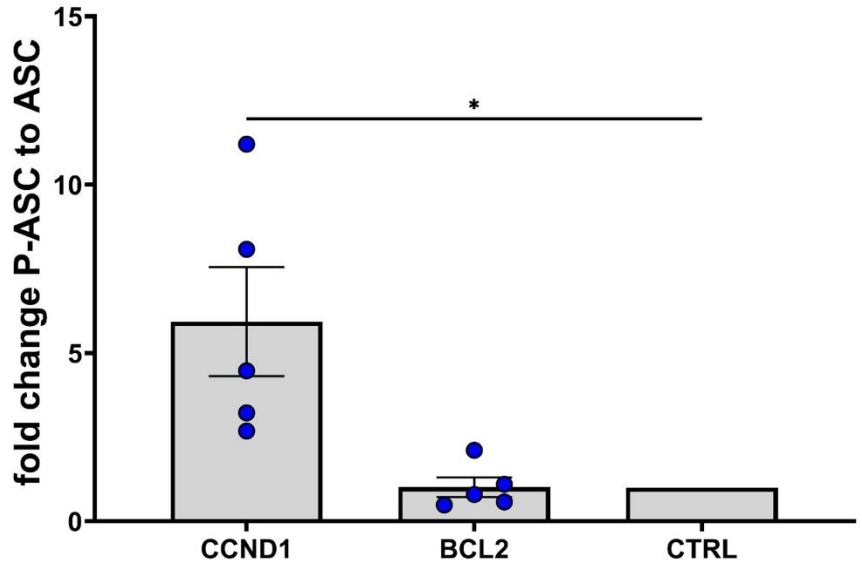

Figure 11: ***Proliferation and apoptotic effect of TNFα and INFγ***

A qPCR (n=5) was employed to observe the gene expression of CCND1 as proliferation marker and BCL2 as a marker for cell apoptosis. CCND1 reached a level of significance (p value 0.016)

4.2.2 Flow Cytometry Annexin V/PI Staining: Differences in Viability

EV from cell culture should come from cells with a viability from at least 90% to avoid isolation of apoptotic bodies or cell debris in the further process (Théry, et al., 2018). Therefore, an Annexin V/PI Staining Assay of the ASCs was made. (Philippé et al., 2002) (Figure 12). P-ASC showed a median viability of 96,3% and ASC a viability of 91,8%. A significant difference between those groups was not given (p-value > 0,05). The assay exposed also the percentage of necrotic and late apoptotic cells. No statistically relevant difference was shown between P-ASC and ASC. As Figure 12 points out, the apoptotic cells show a significant difference between P-ASC and ASC (p-value: 0,03), which could indicate that TNFα and INFγ have a protective effect in the first 48 hours in serum free cell culture conditions.

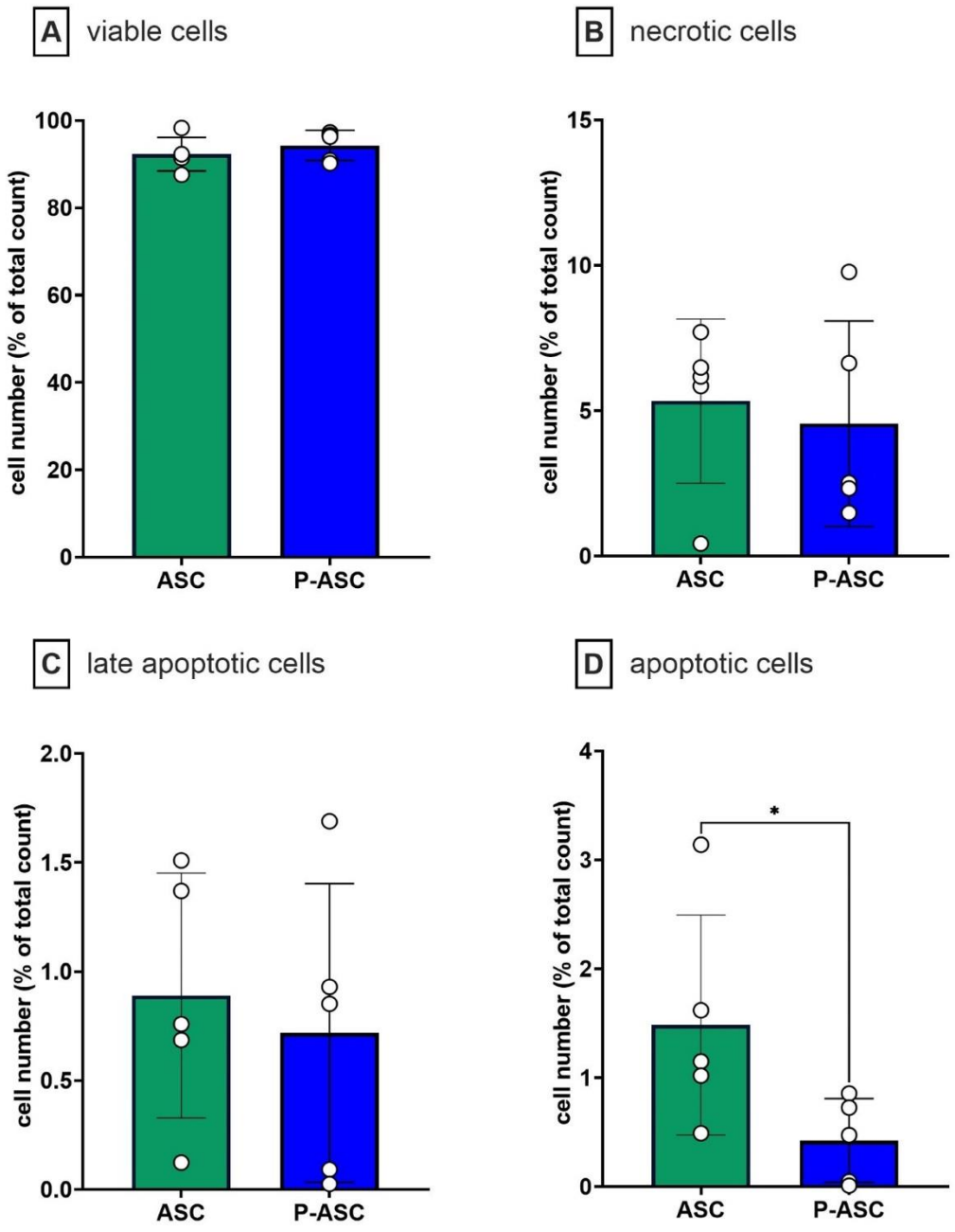

Figure 12: ***Annexin V/PI Staining by Flow Cytometry***

(A) viable cells: ASC: 91,8% P-ASC: 96,3% (B) necrotic cells: ASC: 6,2% P-ASC: 2,5% (C) late apoptotic cells ASC: 0,8% P-ASC: 0,9% (D) apoptotic cells: ASC: 1,2% P-ASC: 0,5% (n=5)

4.2.3 Phase-contrast Microscopy: Morphology

Cell morphology was monitored during in vitro cultivation by phase-contrast microscopy. Figure 13 provides exemplary pictures of the cells after 48h in serum free media. P-ASC have been primed before with TNFα und INFγ for 24 h. ASC appear in typical slim, spindle-like shape, while P-ASC seem to lose that shape,

appear more elongated and show a halo around the nucleus. All five donors showed the same morphology changes throughout the priming.

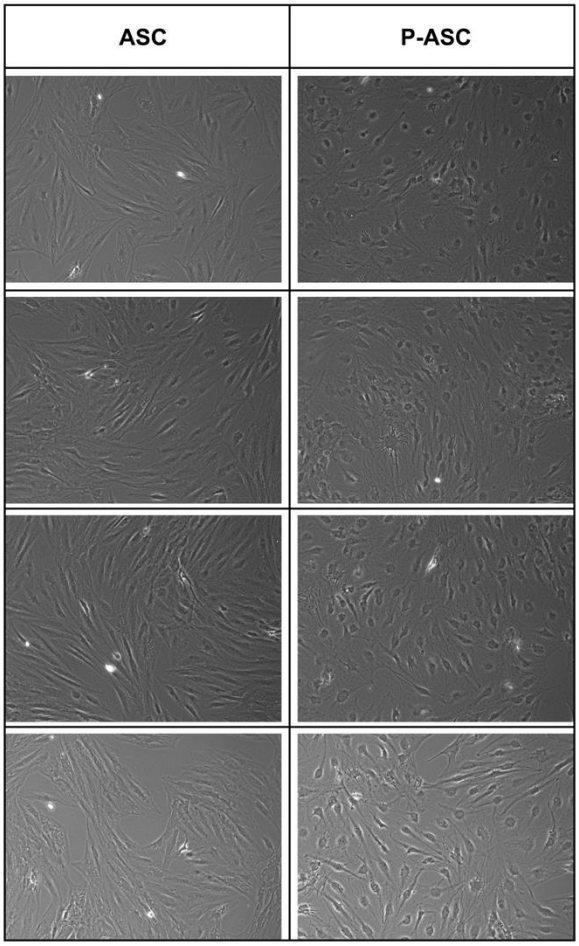

Figure 13: ***Representing pictures of ASC and P-ASC after 48h in serum free culture conditions***

Phase-contrast pictures were taken with an Axioxam MRm camera mounted in an AxioObserver microscope (Carl Zeiss, Jena, Germany;10x magnification, (n=4)). ASC appear in typical slim and spindle-like shape. P-ASC appear more elongated and with a halo around the nucleus

4.3 Characterisation of EV

4.3.1 Nanoparticle Tracking Analysis: Size Distribution and Concentrations

To confirm that the isolated particles fall in the size range of small EV and Exosomes (Théry et al., 2018) a Nanoparticle Tracking Analysis (NTA) was conducted (Figure 14).

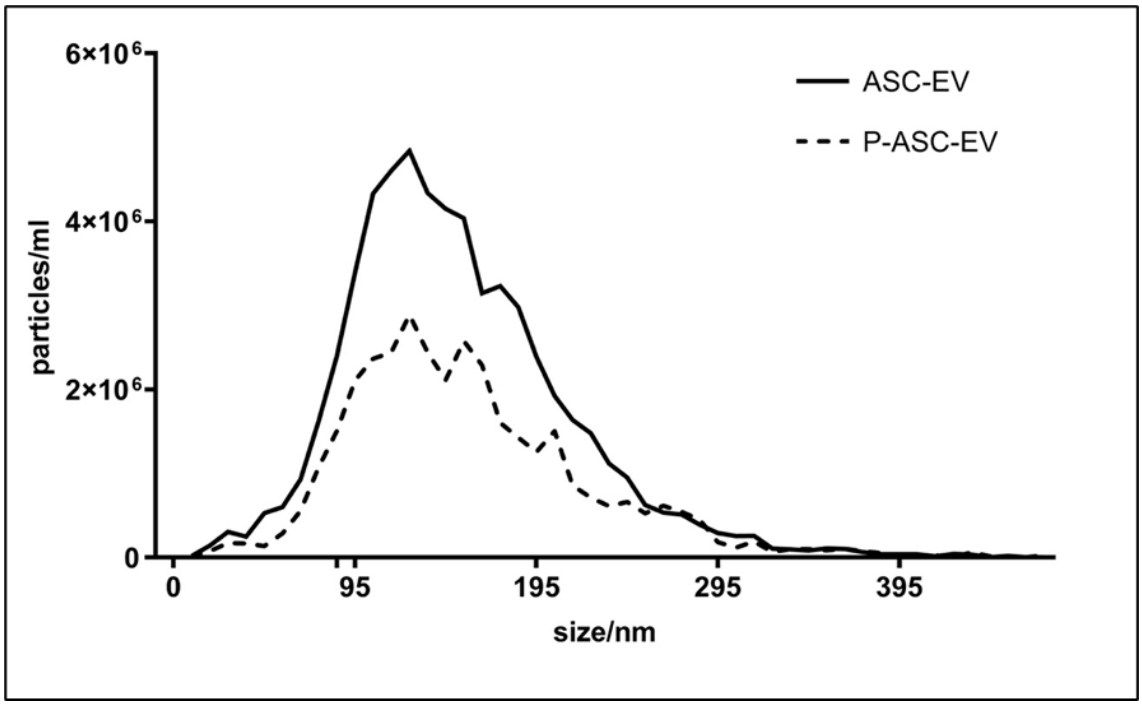

Figure 14: ***NTA size distirubtion***

EV from ASCs showed a median size of 144,4nm. EV from P-ASC showed a median size of 147,5nm. (n=5)

Additionally, the NTA was used to compare the concentrations of particles isolated from either the P-ASC medium or the not stimulated ASC medium. Figure 14 points out that the predominant fraction of particles from both groups and from all five donors are in the needed size range under 200nm.The median diameter from the ASC-EV was 144,3nm and from the P-ASC-EV 147,5nm. The EV from the ASC had their median peak of concentration at 126,1nm and the EV from the P-ASC at 133nm as you can see in Figure 15. Subsequently the highest concentrations of the particles were in the size range of Exosomes.

We could see a lower median concentration from the P-ASC-EV as Figure 15 indicates. However, no statistically significant differences were observed in terms

of size or concentration when comparing EV produced by P-ASC to those produced by ASC.

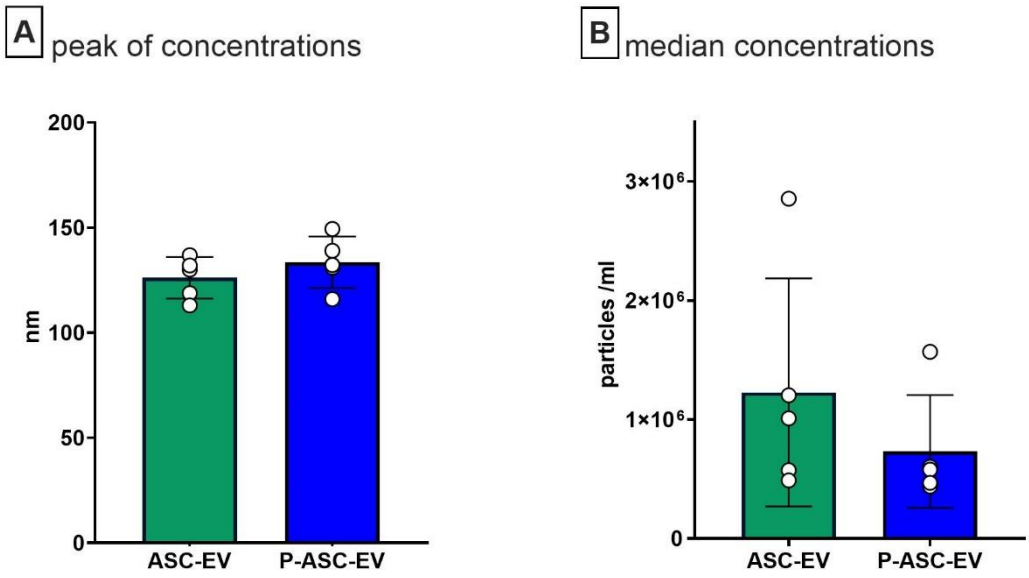

Figure 15: ***peak and median of concentration***

(*A) peak of concentration: ASC-EV had their peak of concentration at 126nm diameter. P-ASC showed the peak of concentration at 133nm. (B) median concentration: Overall the ASC-EV had a median concentration of 1x10E6 particles/ml, the P-ASC-EV presented a median concentration of 0,73E6. (n=5)*

4.3.2 Flow Cytometry: Expression of EV Surface Markers

Flow Cytometry was used, to indicate the presence of the EV-surface markers. Figure 16 shows that CD81 as well as CD63 had a higher expression of the markers compared to the control. A consistently higher or lower mMFI between ASC-EV and P-ASC-EV was not observed. Overall, the NTA and Flow Cytometry combined suggest that EV were separated from the conditioned medium by the PEG enrichment process. A relevant modification of the EV was not observed due to the stimulation of the ASC.

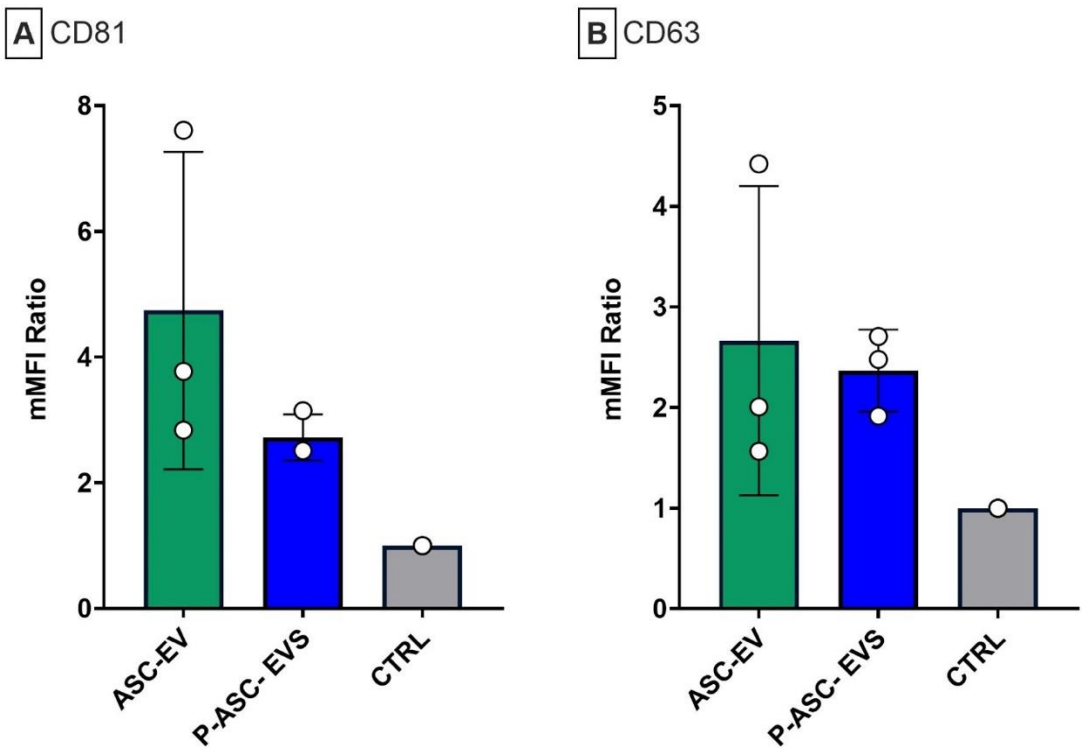

Figure 16: ***Expression of surface markers on EV via beads based Flowcytometry***

A) CD81: A higher (mMFI Ratio: ASC-EV:4,75; P-ASC-EV:2,72) expression of CD81 is shown on ASC-EV as well as on P-ASC-EV, compared to the control. (n=3) (B) CD63: Also, CD63 was more expressed on EV from ASC and P-ASC compared to the control. (mMFI Ratio: ASC-EV:2,67; P-ASC-EV:2,40) (n=3); (mMFI: multiplicity of mean fluorescence intensity).

4.4 Functional Assay: Effect on chondrocytes when treated with ASC-EV or P-ASC-EV

After the separation and the characterization of the EV, the aim of this study was to look at the effect those EV have on inflamed cartilage. Therefore, chondrocytes were isolated from one human donor, cultivated in vitro and were treated with ILß to mimic the environment of osteoarthritic cartilage. Finally, the chondrocytes were treated with ASC-EV or P-ASC-EV for 24h to allow full interaction between cells and EV.

4.4.1 Quantitative PCR : Impact on Gene Expression

To detect whether there is a difference between the interaction of P-ASC-EV with chondrocytes to the interaction ASC-EV have with the chondrocytes, a qPCR was employed. A significant upregulation was observed in *BCL2* (p=0,04) and *CCND1* (p=0,03) due to the previous priming of the cells. Other genes did only lead to a minimal upregulation of the matching gene expression, when chondrocytes were treated with P-ASC-EV. This suggests that in our setup the priming of the initially ASC has no strong impact on the gene expression level of chondrocytes, except for *BCL2* and *CCND1*.

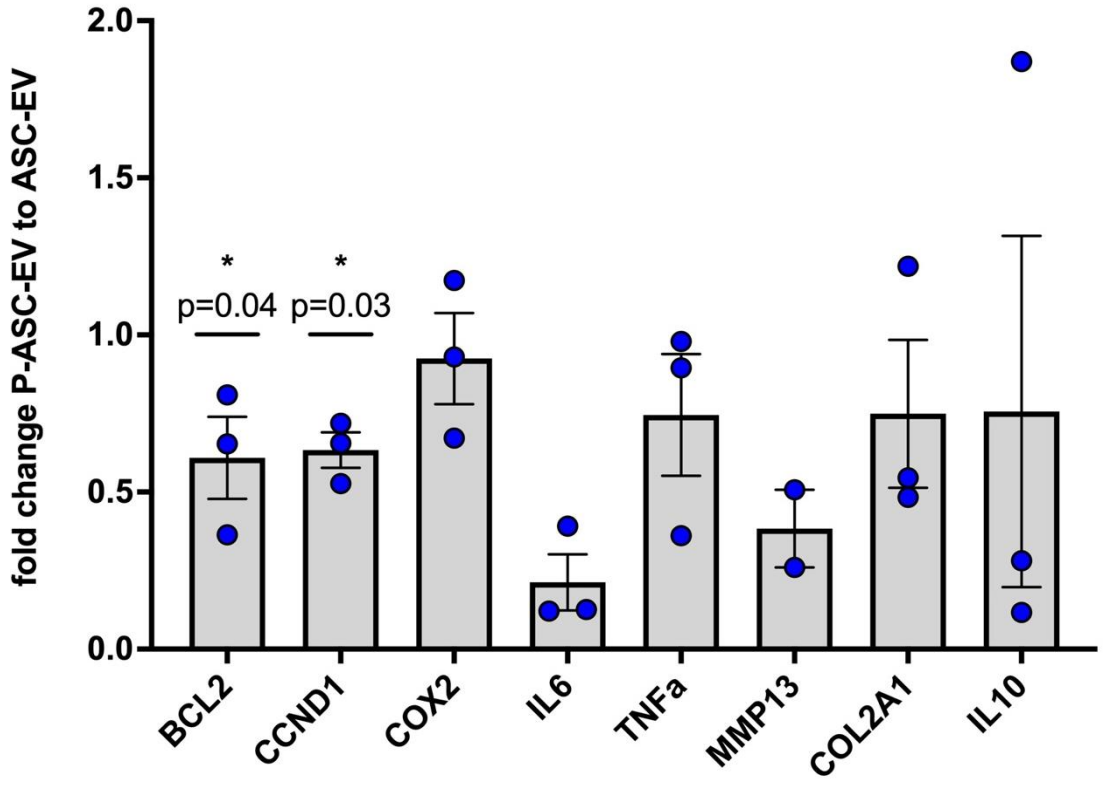

Figure 17: **qPCR of treated chondrocytes**

A significant downregulation was observed in BCL2 (p=0,04) and CCND1 (p=0,03) due to the previous priming of the cells. Other genes did not show a significant up or down regulation. (COX2: p= 0,63; IL6: p= 0,63; TNFa: p= 0,26; MMP13: p= 0,1; COL2A1: p= 0,35; IL10:p=0,69) (n=3; MMP13: n=2)

4.4.2 ELISA: Measurement of Cytokines in Cell Culture Medium

Subsequently, an Elisa Assay was preformed to examine the differences in secretion of proteins during the ASC-EV and P-ASC-EV treatment of chondrocytes. Once again, typical proinflammatory cytokines like COX2, IL6 and TNF alpha

were observed. As shown in Figure 18, priming of EV resulted in no notable differences in the secretion of those cytokines. To observe the cell proliferation BCL2 was measured in the cell medium and showed a lower concentration in the medium from chondrocytes treated with P-ASC-EV compared to the median concentration when treated with ASC-EV. In Contrast, an increased LDH concentration was observed in the medium of chondrocytes treated with P-ASC-EV. However, no statistically significant differences were observed.

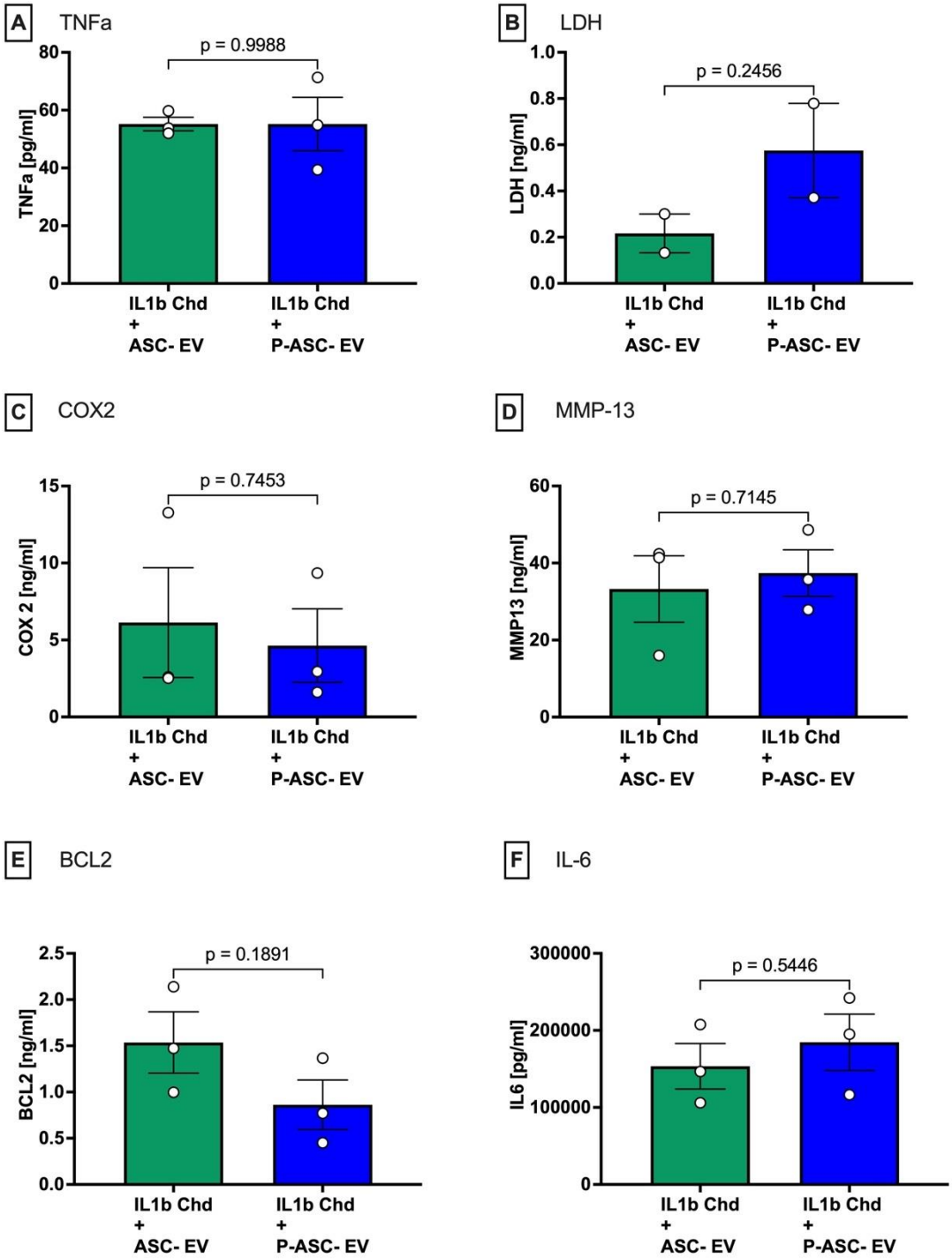

Figure 18: **ELISA-Assay from cell culture medium**

To observe the effect EV from either P-ASC or ASC have on chondrocytes in an inflammatory environment (IL1b), (A) TNFα, (B) LDH, (C) ICOX2, (D) MMP13, (E) BCL2 and (F) IL6 were measured in the conditioned medium. BCL2 concentrations in the cell medium were lower in chondrocytes treated with P-ASC-EV compared to those treated with ASC-EV. An increased LDH concentration was detected in the medium of chondrocytes treated with P-ASC-EV. No statistically outcome were observed in this assay

4.5 Outlook

In 7.4.1 the effect of P-ASC-EV and ASC-EV treatment on the gene expression of chondrocytes was compared. Of course, we also wanted to compare this effect to inflamed chondrocytes, which were not treated with EV and thereby serving as the control. However, due to the limited available material we could only use one sample as a control. Consequently, there is a scientific validity deficiency in these results but still remain noteworthy for future experiments and studies.

4.5.1 Comparative Impact of EV Treatment on Chondrocytes: qPCR Analysis

Again, we tested proinflammatory genes like *COX2*, *TNFα* and *IL10* (Figure 19). All three genes show a noticeable downregulation compared to the control.

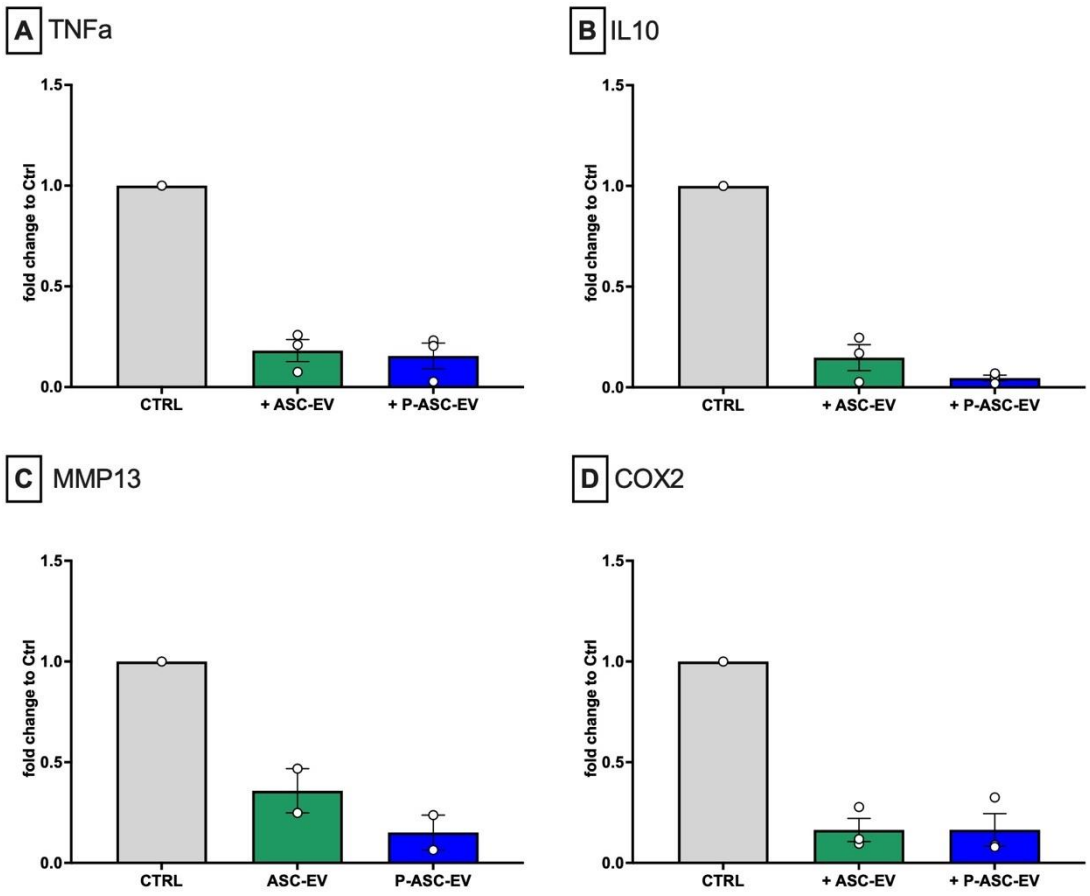

Figure 19: ***Comparative impact of EV treatment on chondrocytes***

A qPCR was performed to compare the gene expression of chondrocytes without EV treatment to chondrocytes treated with EV. Focusing on negative genes in the chondrocytes proliferation and regeneration (A) TNFα, (B) IL10, (C) MMP13, and (D) COX2 were observed. (A)-(D) were all downregulated compared to the control. Due to limited available material, only one sample was utilized as a control. Consequently, no statistical analysis was conducted.

Also, *BCL2* and *MMP13* appear down regulated (Figure 20; Figure 19). To verify the positive effect, we also tested genes which a crucial in vital and proliferative chondrocytes. Interestingly, the results also showed a downregulation of these genes. Which might lead to the assumption, that the EV treatment downregulate the gene expression in general.

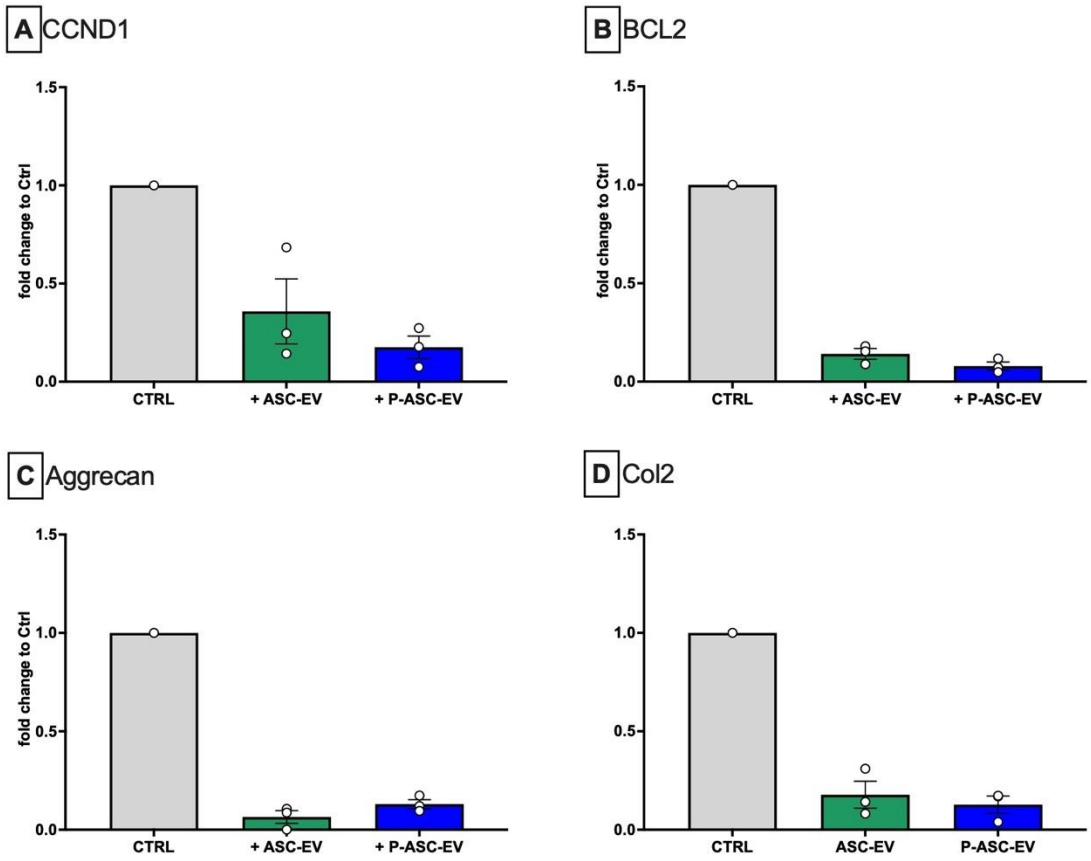

Figure 20: ***Comparative impact of EV treatment on chondrocytes***

A qPCR was preformed to observe the gene expression of inflamed chondrocytes, when not treated with any EV. Focusing now on positive genes in the chondrocytes proliferation and regeneration (A) CCND1, (B) BCL2, (C) Aggrecan, and (D) Col2 were observed. (A)-(D) were also all downregulated com-pared to the control. Due to limited available material, only one sample was utilized as a control. Consequently, no statistical analysis was conducted

5. Discussion

5.1 Enhancing Extracellular Vesicle Seperation: Improved PEG Method

Considering the increasing interest in EV over the past years, numerous methods for EV enrichment and separation have been publicized and are already commercially available (Konoshenko et al., 2018). Thus, finding the right method for your individual set up can be challenging and individual requirements have to be kept in mind.

To preserve the integrity of the EV (Veerman et al., 2021; Jia et al., 2022) and avoid toxicity towards the target cells in the following functional assay (Lang et al., 2022b), we chose the PEG method based on precipitation and added filtration steps to rule out larger EV, apoptotic bodies and cell debris.

Consequently, no special equipment was needed and the execution was straightforward. Nevertheless, several challenges need to be taken into consideration when using PEG for EV separation from cell culture.

Firstly, it is particularly important that attention is paid to the possibility of coisolation (Tian et al., 2020b). Consequently, no serum should be used during the isolation steps (Théry et al., 2018). Even commercially available serum which claims to be EV depleted, seems to lack the necessary purity as we saw in our experiments. These results align with findings reported in other publications (Lehrich et al., 2018;Kaur et al., 2014). It is also important to bear in mind, that many materials needed for other assays contain serum, such as antibodies, and could lead to biased results, due to contamination.

Secondly, the PEG method does not selectively enrich EV of one particular size, therefor an additional separation step is useful when looking for smaller EV and exosomes. By using a filter, EV of a larger size can be sorted out (Rider et al., 2016b).

Thirdly, the volume of the cell culture medium is crucial. Our findings suggest, that scaling up the pooled volume to at least 150ml is necessary to obtain a useful amount of EV when using the PEG method. This aspect again brings several challenges within the process of cell culture and the comparability of results. We avoided any freezing of medium and used only medium directly from the cell culture flask (Wright et al., 2022). Therefore, the medium had to be concentrated which had the positive side effect of another filtration and cleaning step (Lang et al., 2022b).

Overall, we were able to demonstrate that our PEG method enriches extracellular vesicles within the specified size range, as evidenced by NTA. Positive signals for CD81, CD64, and CD9 were observed through flow cytometry, as well as Western blotting.

5.2 Cytokine Priming of Adipose Derived Stem Cells

Mesenchymal stem cells, such as ASC, are widely recognized for their role in the regenerative and anti-inflammatory processes associated with osteoarthritis.

Several clinical studies have demonstrated the positive effects of directly injecting MSC into human joints. For instance, Koh et al. showed that injecting MSC from the infrapatellar fat pad into osteoarthritic knees improved knee function by reducing pain. Similarly, Jo et al. reported a reduction in cartilage defects after injecting MSC into osteoarthritic knee joints, leading to improved knee function. However, there remain challenges regarding the comparability of the studies and the need for long-term evaluations

It has been documented that the immunomodulatory response is mediated not only through cell-to-cell contact but also by the secretion of cellular factors (Zhou et al., 2019).

Recently, there has been a particular focus on the role of EV derived from MSC in this context (Mardpour et al., 2019). The ability of MSC to produce immune-regulatory and regenerative factors appears to be a direct response to an inflammatory environment (Madrigal et al., 2014b). The cytokines TNFα and INFγ play an important role in this by activating MSC and improve their anti-inflammatory response (López-García et al, 2021). Domenis et al. suggest that priming the cells with cytokines like TNFα and INFγ is necessary to induce the release of EV with immunomodulatory capacity and cause macrophages to transform from M1 proinflammatory phenotypes to M2 anti-inflammatory cells (Domenis et al., 2018a). Also, Ragni et al. demonstrated that priming MSC with INFγ resulted in an enhanced capability of EV regarding joint disease (Ragni et al., 2020). It is important to note that the concentrations of TNFα and INFγ in the synovial fluid of OA patients are significantly lower than those used in vitro experiments. The in vivo concentration is measured in pg/ml (INFγ 142,7+/- 73,3 pg/ml; TNFα 5+/- 3,1 pg/ml (Nees et al., 2019)), and even in advanced stadiums of OA, such high concentrations as those used in vitro set ups are not observed.

Therefore, the clinical application of non-primed MSC does not seems to be efficient. Priming the cells before application is necessary, comparing the low concentration of cytokines in vivo.

5.2.1 Impact on Morphology

In this study we observed that the cells reacted to this inflamed environment by changing their morphology from slim, spindle like bodies to more elongated ones with a halo around the nucleus (Figure 13). This change has been reported before by several studies. Prasanna et al noted that despite the observed morphological changes, the mesenchymal markers CD73 and SSEA4 remain unaffected. Furthermore, the ability to differentiate into all three lineages is not affected following pretreatment with TNFα and INFγ (Prasanna et al., 2010). MSC underwent long-term treatment (14-28 days) with TNFα, similar changes in morphology were observed. The authors suggest that TNFα triggers MSC to differentiate into "fully mitotic cells with neuroglial-like morphology" (Egea et al., 2011). While this impact of TNFα and INFγ on morphology is acknowledged, further investigation is required to truly uncover the underlying cause of this morphological change and its impact on the production of EV.

Besides the change of the cell body, we, as other publications, observed the production of fibers in all primed ASC. We interpreted these fibers as stress fibers. Stress fibers are actin filaments, which play an important role in the cytoskeleton (Kassianidou et al, 2015). The production of stress fibers appears to be a reaction to changes in the ECM. The purpose of these fibers is to support the process of differentiate into other cell types, maintain hemostasis, cell migration and adhesion (Wozniak et al, 2009; Blanchoin et al., 2014).

This raises the question whether this reaction may even change the overall purpose and modulate cellular organization. Additionally, it is important to investigate how these stress fibers might influence the biogenesis and production of EV.

5.2.2 Impact on Viability

During the process of programmed cell death cells release apoptotic bodies, which might be mistakenly include in the count of EV (Frey & Gaipl, 2011). Therefore, the International Society of Extracellular Vesicles strongly recommends ensuring a high percentage of viable cells during the harvest process (Théry et al., 2018). Primed MSC only tolerate an individual threshold of cytokine concentration, before they initiate the apoptotic pathway (Barrachina et al., 2017). We did not observe any significant difference in the viability of P-ASC compared to ASC (Figure 12), suggesting that the used concentration of cytokines as well as the period of time of treatment had no effect on cell viability. Interestingly there was a significant difference between the number of early apoptotic cells after treatment. It appears that priming at this concentration protects ASC to go in to apop-

tosis. It is reported that TNFα exhibits a paradoxical effect by inducing both apoptosis and proliferation through different receptors, TNFR1 and TNFR2. Both transmembrane receptors are expressed on MSC (Yan et al., 2018;Beldi et al., 2020). On the other Hand, Böcker and colleagues did not see any antiapoptotic effect by priming MSC with 50ng/ml TNFα (Böcker et al., 2008).

5.2.3 Impact on Gene Expression

To investigate the impact of cytokines on the gene expression level of ASC, we conducted a RT PCR analysis. INFγ and TNFα are known for their potential to increase the expression of inducible nitric oxide synthase (*iNOS)* in MSC (Burke et al., 2013;Egea et al., 2011). *iNOS* plays a crucial part in the immunomodulatory potential of MSC (Sato et al., 2007). This aligns with our results of a significant upregulation of *iNOS* after priming the ASC with TNFα and INFγ (Figure 10). In contrast to Barrachina et al we did not observe a significant increase of *TNFα*. Also, *IL6* and *COX2* showed only a trend for upregulation*,* even though it has been reported that the protein concentration in conditioned medium of pretreated MSC is significantly higher (H.-M. Yang et al., 2018;Hemeda et al., 2010). Crop et al reported a similar upregulation of *COX2 and IL6* (Crop et al., 2010).

Another aspect of interest is the impact pretreatment has on cell survival and proliferation. TNFα is known for simulating proliferation of MSC up to an individual concentration, while INFγ can cause growth suppression (Chan et al., 2008; Prasanna et al., 2010). Egea and colleagues could show that 5µg/ml TNFα doubled the cell proliferation measured by WST-1 assay (Egea et al., 2011). On the other hand, a cytokine mixture of TNFα and INFγ suppressed proliferation measured by trytpan blue exclusion assay (Domenis et al., 2018a). Crop et al reported as well that the cell number decreased after treatment with TNFα, INFγ and IL6 (Crop et al., 2010).

In this study our aim was to examine the proliferation and cell survival by comparing the gene expression of *BCL2* and *Cyclin 1* (*CCND1)* with our control. *BCL2* is an antiapoptotic gene, involved in the mitochondrial apoptosis pathway (Hotchkiss et al., 2009). Interestingly, Barrachina and colleagues demonstrated, that the expression of *BCL2* appears to be dependent on the cytokine concentration. A concentration of 20ng/ml (TNFα+ INFγ) suppressed *BCL2* expression, while a concentration of 50ng/ml upregulated the gene. In our study, we observed a slight trend towards downregulation of *BCL2*, consistent with our result of the Annexin V/PI Staining. *CCND1* is a regulatory protein involved in the cell cycle, and it has been reported that TNFα upregulates *CCND1,* thereby stimulating the proliferation of MSC (Böcker, et al., 2008). These finding aligns with our results, which show a significant upregulation of *CCND1* (Figure 11).

Even though primed MSC are in the focus of research for a long time, literature is partially contradictory. The comparability of results is influenced by variations in treatment duration, cytokine concentration and adding another cytokine. It is worth noting that cells in this study underwent 48h in serum free medium condition after the treatment and before being analyzed. However, overall, we observed a significant change in morphology without affecting cell viability. Additionally, cytokines exhibited a proinflammatory impact on ASC gene expression, along with significantly stimulating proliferation and an antiapoptotic effect.

5.3 EV - Characterisation and Analysis

As noted earlier, primed MSC exhibit a greater influence on target cells compared to those without pre-treatment. One way they exert this influence is by EV. Therefore, the aim of this study was to investigate whether size, concentration and surface markers of EV change after ASC being primed with TNFα and INFγ. Additionally, considering the clinical approach of EV in the future, it is crucial to understand methods to increase EV secretion as well as methods to improve harvest of EV (Debbi et al., 2022). Several factors, such as hypoxia (Zeng et al., 2021), acidity (Logozzi et al., 2017) and hypoglycaemia (Rice et al., 2015) could lead to a higher yield of EV. Interestingly, also serum deprivation can lead to higher EV production (Haraszti et al., 2019). On the other hand, it appears that MSC-treatment with cytokines like TNFα and INFγ (R. Yang et al., 2020;Domenis et al., 2018), as well as the protein LPS (Ti et al., 2015;Fiedler et al., 2018), has no effect on EV numbers.

While our concentration results of 1x10E6 particles/ml from ASC and 0,73x10E6 particles/ml from P-ASC may comparatively be lower than other reported concentration, there was also no significant changes of size or concentration due to the treatment with TNFα and INFγ (Figure 14),(Figure 15). Nevertheless, some studies have reported an increased EV release resulting from priming the cells like de Pedro et al. (de Pedro et al., 2021). It is important to note that in this particular study, a 4 times higher cytokine concentration of 100ng/ml was utilized. Consequently, comparisons are challenging due to variations in enrichment methods, cell medium components, medium volume and different methods for quantification of EV.

To characterize the EV, we employed a bead-based flow cytometry. Our primary focus was to characterize the EV, followed by comparing the expression of surface markers. As Figure 16 points out, both groups presented the surface markers CD63 and CD81 and therefore can be characterized as extracellular vesicles (Théry et al., 2018). Consistent with prior studies, we detected no changes of

surface markers resulting of priming the ASC (de Pedro et al., 2021;Zhang et al., 2018).

5.4 Investigating the Therapeutic Potential of P-ASC-EV on inflamed Chondrocytes

For many years now, MSC have been employed in the context of OA. Two primary factors make them intriguing for therapeutic approach. Firstly, their ability to transform into chondrocytes (Prockop, 1997; Pittenger et al., 1999). The chondrogenic potential of ASC appears to be lower compared to other mesenchymal stem cells, such as bone marrow stem cells and synovium stem cells (Sakaguchi et al., 2005; Danisovic et al., 2009). On the other hand, ASC provide a higher capacity for proliferation then BMSC (Lotfy et al., 2014). Nevertheless, since the early 2000s, ASC have been widely used as a source of chondrocytes and for tissue engineering (Rada et al., 2009). Secondly, their impact of the immunomodulation by paracrine activity. This involves their ability to mediate anti-inflammatory, anti-apoptotic, angiogenic, mitogenic and anti-fibrotic responses (Caplan, 2007). Upon recognizing that all cells, including MSC, produce EV for cell-to-cell-communication, the examination of the role of MSC-derived EV has become a focal point in numerous OA studies. For example, Woo et al could show that, in vitro, ASC derived EV increased proliferation of chondrocytes in an EV-dose-dependent manner. Additionally, these EV were found to suppress catabolic markers such as *MMP1*, *MMP3*, *MMP13* and *ADAMSTS-5* and increased *collagen II* expression in chondrocytes (Woo et al., 2020).

Knowing that primed MSC have a more effective way to reduce inflammatory response (Domenis et al., 2018b), another objective of our study was to investigate the effect EV from primed ASC have on inflamed chondrocytes compared to EV from non-primed ASC.

Therefore, chondrocytes inflamed with IL1ß were exposed to EV at a concentration of $50x10^6$ particles/ml derived from ASC or P-ASC for 24 hours. We employed a qPCR to examine the gene expression of key factors in OA. As Figure 17 points out, the expression of key genes associated with the cell cycle, such as *CCND1* and *BCL2,* changed significantly following the priming of the ASC. In fact, both BCL2 and CCND1 were downregulated, indicating that the treatment of chondrocytes with P-ASC-EV result in a dysfunctional cell regulation.

On the other hand, genes commonly expressed in an OA environment like *COX2*, *IL6*, *TNFα*, and *MMP13,* did not exhibit any notable changes. Additionally, *IL10*, recognized for its ability to inhibit proinflammatory responses (Schulze-Tanzil et al., 2009) and *COL2A1,* known as a protective factor of chondrocytes (Carballo

et al., 2017), showed no relevant difference due to the priming of ASC. For further investigations, we also conducted an ELISA assay of the supernatant. The results did not show any significant changes in the secretion of proinflammatory cytokines following the priming of ASC; however, align with PCR analysis, which indicates a lower concentration of BCL2 in the medium of P-ASC-EV and a higher concentration of LDH.

This observation suggests, in this experimental setup, the priming of the ASC did not influence the chondrocytes in terms of inflammatory response, but had a negative effect on cell-cycle regulation. Interestingly, this is contrasts with the antiapoptotic effect that priming has on ASC themselves (see 8.2.3.).

However, comparing this experimental setup to other published studies, there a several factors that may have contributed to these results.

There are three published studies with a comparable in vitro setup (Table 26). Firstly, there is variation in the stimulation of the source MSC regarding the type of cytokine (IL1ß, TNFα, INFγ, TGFb, INFa) as well as the concentration. For instance, Nguyen et al used a ten times higher concentration of INFγ then we applied. Also, our study is, to the best of our current knowledge, the first to investigate a TNFα+ INFγ mixture to stimulate ASC for an EV treatment on chondrocytes in vitro.

Secondly, it is important to note that the used amount of EV is in these studies are quantified by proteins per ml. Théry et al discuss this, noting potential higher results due to co-isolation of other proteins and increased concentration resulting from disrupted EV prior to the analysis assay. Consequently, it stays unclear how many particles or actual EV were applied, when using proteins per ml for quantification of EV.

Last, and most important, variations exist in the duration of treatment of chondrocytes with EV. It is reported that within 24 hours chondrocytes absorb EV (Jammes et al., 2023). Interestingly, Cavallo et al demonstrated that gene expression of OA related genes was upregulated after 4 hours but at 15h again reduced. On the other hand, they showed that released factors increased after 15hours compared to the 4 hours' time point. In contrast, the authors of the other comparable studies observed significant changes in gene expression only after 7 days and 14 days.

These findings imply, that in our experimental setup, the 24 hours analysis may be both, too early and too late to detect significant up or downregulation of proinflammatory genes. And on the other hand, induce already cellular stress. Unlike Cavallo et al, we did not examine significance in released factors measured with ELISA assay. There is also the possibility that the quality of the cartilage cells

used was insufficient. Given the lack of other cartilage donors, these results should be interpreted with caution. For future experiments, it is recommended to evaluate cartilage cells from multiple donors to avoid this potential source of error. We additionally compared our findings with chondrocytes that did not undergo EV treatment. However, limited material allowed for only one control duplicate. Consequently, there is a scientific validity deficiency in this dataset. But it is still noteworthy that overall, these results imply a downregulation not only form OA supporting genes, but also from protective chondrocyte genes. This leads to the assumption that the gene expression in general was downregulated by the EV treatment, regardless of the pretreatment of the ASC.

In conclusion, our final experiments suggest that using EV from ASC rather than P-ASC may have a more positive impact on chondrocytes. For future studies, we suggest to extend the duration of the treatment, use multiple donors for chondrocytes, perform chondrocyte analyzes at different time points and assess the viability and health of chondrocytes. Furthermore, it would be interesting to investigate the possible differential influence the quantified number of EV from primed ASC have on chondrocytes in vitro. This would be a crucial step to standardize OA treatment protocols in vitro, ensuring the reproducibility of results. Additional this standardization is essential for clinical applications.

To gain a comprehensive understanding of the full spectrum of priming effects on EV, an insightful approach would be to perform EV proteome analyses before the functional chondrocyte assay.

	Nguyen et al., 2022	**Jammes et al., 2023**	**Cavallo et al., 2022**
Source of MSC	Human Umbilical cord MSC	Equine Bone Marrow MSC	Human ASC
Cytokines	TGFb (10 ng/mL), IFNa (20 ng/mL), or TNFα. (20 ng/ml)	IL-1β(20 ng/mL), TNF-α (10ng/mL), IFN-γ(100 ng/mL)	IL1ß (2ng/ml)
Used amount of EV	10µg/ml proteins BCA-Assay	6µg//ml proteins 0,5µg/ml proteins micro BCA-Assay	10µg/ml proteins NanoOrange Protein Quantification Kit
Duration of EV treatment	7 days	14 days	4 hours /15 hours

Table 26: Experimental Setups of comparable in vitro studies

6. Conclusion

6.1 Overview of Study Findings

In summary, the outcomes of this study provide the following insights:

- The modified PEG method, including an additional filtration and volume concentration setup, successfully enriched EV.
- The priming of ASC with 25ng/ml TNFα + 25ng/ml INFγ was demonstrated not to affect cell viability. However, it strongly influenced cell morphology and exhibited a trend toward promoting proliferation and an anti-apoptotic effect.
- Priming ASC had no noteworthy impact on EV concentration, size or expressed surface markers.
- There was no difference in effect on chondrocytes between EV from P-ASC or ASC.

6.2 Recommendations for Future Experiments

To overcome challenges related to comparability, limited materials, significant timepoints and effective treatment, we propose the following considerations:

- The suitable enrichment method should be chosen based on the available material, considering subsequent EV analysis and the type of functional assay to be conducted.
- Cell culture should be standardized and the use of serum, such as FBS, should be avoided before the enrichment method.
- The volume of cell culture medium is crucial for determining EV numbers. Volume should be scaled up to a reasonable level as it is essential to generate sufficient material for comprehensive EV characterization and for the implementation of adequate functional assays.
- We recommended to evaluate cartilage cells from multiple donors to avoid donor specific reactions
- Our findings suggest to extend the duration of EV treatment on chondrocytes and measure treatment effects at different time points.

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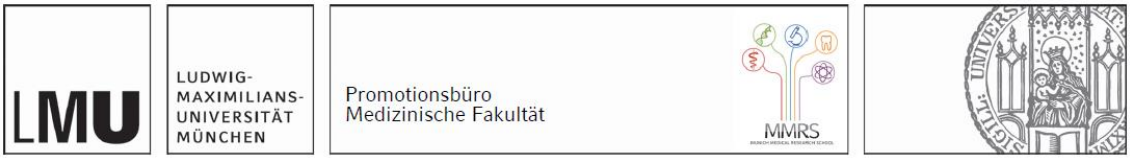
9. Affidavit



Eidesstattliche Versicherung

Erb, Anouk

Ich erkläre hiermit an Eides statt, dass ich die vorliegende Dissertation mit dem Titel:

Extracellular Vesicles of Primed Adipose Derived Stem Cells as a Therapeutic Approach for Osteoarthritis

selbständig verfasst, mich außer der angegebenen keiner weiteren Hilfsmittel bedient und alle Erkenntnisse, die aus dem Schrifttum ganz oder annähernd übernommen sind, als solche kenntlich gemacht und nach ihrer Herkunft unter Bezeichnung der Fundstelle einzeln nachgewiesen habe.

Ich erkläre des Weiteren, dass die hier vorgelegte Dissertation nicht in gleicher oder in ähnlicher Form bei einer anderen Stelle zur Erlangung eines akademischen Grades eingereicht wurde.

München,08.10.2025 Erb,Anouk

Ort, Datum Unterschrift Doktorandin

10. Confirmation of Congruency

Confirmation of congruency between printed and electronic version of the doctoral thesis

Erb, Anouk

Surname, first name

I hereby declare that the electronic version of the submitted thesis, entitled:

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is congruent with the printed version both in content and format.

München, 08.10.2025 — Place, Date

Erb,Anouk — Signature doctoral candidate