Optimizing the Immunoregulatory Properties of Human Mesenchymal Stromal Cells and their Extracellular Vesicles by Immunological Priming

Cumulative Doctoral Thesis

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Submitted by

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"Science does not present itself to human until mind conquers matter in striving to subject the result of experimental investigation to rational combinations."

Alexander von Humboldt

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I. Abbreviations

ADIRF	Adipogenesis Regulatory Factor
AKAP12	A-kinase anchoring protein 12
Ang-1	Angiopoietin-1
APC	Allophycocyanin
AS	Autologous Serum
B7-H4	B7 Homolog 4
BDNF	Brain-Derived Neurotrophic Factor
bFGF	basic Fibroblast Growth Factor
BP	Biological Processes
BSA	Bovine Serum Albumin
BV421	BrilliantViolet421™
CCL-2, 3, 4, 5, 6, 20	CC Motif Chemokine Ligand 2, 3, 4, 5, 6, 20
CD	Cluster of Differentiation
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
COL1A1	Collagen Type I Alpha 1
COL1A2	Collagen Type I Alpha 2 chain
CPDye	Cell Proliferation Dye
CXCL-2, 3, 5, 10, 11, 12	CXC Motif Chemokine Ligand 2, 3, 5, 10, 11, 12
DAMP	Damage-Associated Molecular Patterns
DAVID	Database for Annotation, Visualization, and Integrated Discovery
DC	Dendritic Cells
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
EIF2AK2	Eukaryotic Translation Initiation Factor 2-Alpha Kinase 2
ELISA	Enzyme-Linked Immunosorbent Assay
FACS	Fluorescence-Activated Cell Sorting
FITC	Fluorescein Isothiocyanate
FBS	Fetal Bovine Serum
FSC	Forward Scatter
G-CSF	Granulocyte Colony-Stimulating Factor
Galectin-1	Galectin-1
GDNF	Glial Cell Line-Derived Neurotrophic Factor
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
GO	Gene Ontology
HGF	Hepatocyte Growth Factor
HLA-A	Major Histocompatibility Complex, Class I, A
HLA-G	Human Leukocyte Antigen G
HO-1	Heme Oxygenase-1
IDO	Indoleamine 2,3-Dioxygenase
IFN-γ	Interferon-gamma
IFN-γ-R	Interferon-gamma Receptor
IFN-beta	Interferon-beta
IFN-I	Interferon Type I
IGF	Insulin-like Growth Factor

IL-1β	Interleukin-1 beta
IL-1β-R	Interleukin-1 beta Receptor
IL-6	Interleukin 6
IL-8	Interleukin 8
IL-10	Interleukin-10
KGF	Keratinocyte Growth Factor
LIF, MIF	Leukemia Inhibitory Factor, Macrophage Migration Inhibitory Factor
M-CSF	Macrophage Colony-Stimulating Factor
MMP-2, 9	Matrix Metallopeptidase 2, 9
MVB	Multivesicular Body
NGF	Nerve Growth Factor
PAGE	Polyacrylamide Gel Electrophoresis
PDGF	Platelet-Derived Growth Factor
PD-L1, PD-L2	Programmed Death-Ligand 1, 2
PGE2	Prostaglandin E2
PHB2	Prohibitin 2
PMN	Polymorphonuclear Neutrophils
PTEN	Phosphatase and Tensin Homolog
PVDF	Polyvinylidene Difluoride
Rb	Rabbit
RIPA	Radioimmunoprecipitation Assay Buffer
ROS	Reactive Oxygen Species
SAMHD1	SAM and HD domain containing deoxynucleoside triphosphate triphosphohydrolase 1
SARS1	Seryl-tRNA synthetase 1
SEMA3A	Semaphorin-3A
SERPINB2	Serpin Family B Member 2
SOD2	Superoxide Dismutase 2
SSC	Side Scatter
STAT1	Signal Transducer and Activator of Transcription 1
STAT3	Signal Transducer and Activator of Transcription 3
STRING	Search Tool for the Retrieval of Interacting Genes
TBS	Tris Buffered Saline
TGF-β	Transforming Growth Factor Beta
TIMP-1, 2	Tissue Inhibitor of Metalloproteinases 1, 2
TLR 3	Toll-Like Receptor 3
TNF-α	Tumor Necrosis Factor alpha
TNF-α-R	Tumor Necrosis Factor alpha Receptor
Tregs	Regulatory T Cells
TSG-6	TNF-Stimulated Gene 6
TWEEN	Polyoxyethylene Sorbitan Monolaurate
Type 1 IFN	Type 1 Interferon
VEGF	Vascular Endothelial Growth Factor

I. List of Figures

Figures of the citated cumulative first-author publications

Hackel et al., 2021:

Hackel et al., 2023:

Figures of Supplemental Report

II. List of Tables

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1. Abstract

1.1 Summary

This cumulative dissertation addresses the immunoregulatory properties of mesenchymal stromal cells (MSCs) and their extracellular vesicles (EVs). The research elucidates the molecular responses by MSCs when exposed to a multitude of inflammatory cytokines, including tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), and interferon-gamma (IFN-γ), thereby contributing to a deeper understanding of MSCs' immunoregulatory function. In the initial study, we compared the effects of TNF-α, IL-1β, and IFN-γ on MSCs, focusing on the augmented immunoregulatory response towards polymorphonuclear neutrophils (PMN). Our findings reveal a critical feedback loop where TNF-α and IL-1β enhance the expression of interferon-gamma receptor (IFN-γR) via nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NF-κB) signalling. This upregulation augments MSC responsiveness to IFN-γ, fostering an increased Interleukin 8 (IL-8) release and promoting the recruitment of PMN. Building upon this, the following study assessed the immunoregulatory properties of MSCs and their EVs, to enhance the immunosuppressive effects towards activated T cells and foster the induction of regulatory T cells in a (programmed cell death protein 1) PD-1 dependent manner. We demonstrated that EVs derived from primed MSCs not only attenuate the severity of graft-versus-host disease in a murine model but also prolong survival. In conclusion, this dissertation unveils priming strategies to potentiate the immunoregulatory functions of MSCs and their EVs, offering promising opportunities for the development of more effective MSC-based therapies.

1.2 Zusammenfassung

Diese kumulative Dissertation befasst sich mit den immunregulatorischen Eigenschaften von mesenchymaler Stromazellen (MSCs) und ihrer extrazellulären Vesikel (EVs). Die Studien bieten Einblick in die molekulare Antwort von MSCs bei der Exposition gegenüber verschiedenen pro-inflammatorischen Zytokinen, darunter Tumornekrosefaktor-alpha (TNF-α), Interleukin-1 beta (IL-1β) und Interferon-gamma (IFN-γ). Dies trägt zu einem tieferen Verständnis der immunregulatorischen Funktion von MSCs bei. Im ersten Abschnitt verglichen wir die Auswirkungen von TNF-α, IL-1β und IFN-γ auf MSCs. Unsere Ergebnisse zeigen eine verstärkende Rückkopplung, bei der TNF-α und IL-1β die Expression des Interferon-Gamma-Rezeptors (IFN-γR) über den Nuklearfaktor "Kappa-Light-Chain-Enhancer" aktivierter B-Zellen (NF-κB) erhöhen. Durch diese Hochregulierung wird die Sensitivität von mesenchymalen Stammzellen gegenüber IFN-γ erhöht, was eine verstärkte Freisetzung von IL-8 bewirkt und die Rekrutierung von polymorphkernige Neutrophile (PMN)

fördert. Darauf aufbauend wurden in der folgenden Studie die immunregulatorischen Eigenschaften von MSCs und ihren EVs untersucht, um die immunsuppressive Wirkung auf aktivierte T-Zellen zu verstärken und die Induktion regulatorischer T-Zellen in Abhängigkeit von PD-1 zu fördern. Wir konnten zeigen, dass EVs aus konditionierten MSCs nicht nur den Schweregrad der Transplantat-gegen-Wirt-Krankheit (GvHD) in einem Mausmodell vermindern, sondern auch das Überleben verlängern. Zusammenfassend lässt sich sagen, dass die Studien eine Konditionierungs-Strategie zur Potenzierung der immunregulatorischen Funktionen von MSCs und ihren EVs aufzeigt, welche vielversprechende Möglichkeiten für die Entwicklung effektiverer MSC-basierter Therapien bietet.

2. Introduction to the Theoretical Background

2.1 Mesenchymal Stromal Cells

Approximately fifty years ago, Friedenstein successfully isolated fibroblast-like and colony-forming cells from bone marrow, demonstrating their ability to regenerate bone fragments and support haematopoiesis in vivo (Friedenstein, 1980). Subsequent research on the multilineage differentiation potential of mesenchymal stromal cells (MSCs) gave rise to a controversy surrounding their stemness (Caplan, 1991). In recent years, the investigation of MSCs' immunomodulatory properties has highlighted their significance as an integral component of the immune system (Pittenger et al., 1999;Dominici et al., 2006b).

2.2 Technical Definition of MSCs

Despite more than four decades since the discovery of MSCs, the exact definition and biological function of these cells remain subjects of ongoing debate. The challenge in defining MSCs primarily arises from their localization within various tissues, where they constitute only a small fraction, approximately 0.1%. Friedenstein initially demonstrated that transplanted heterogeneous fractions of bone marrow-derived MSCs differentiate into bone, cartilage, and adipose tissues. He referred to these cells with their multi-differentiation potential as postnatally skeletal stem cells, which establish a supportive cellular niche for hematopoietic stem cells (HSCs). Several decades later, development of an in vitro differentiation system brought renewed attention to MSCs, suggesting their potential to differentiate into various tissues, including smooth muscle, tendons, ligaments, and even neuronal tissue (Pittenger et al., 1999), **(Figure 1)**. However, these in vitro differentiations were primarily driven by mixtures of growth factors and lacked validation in in vivo models.

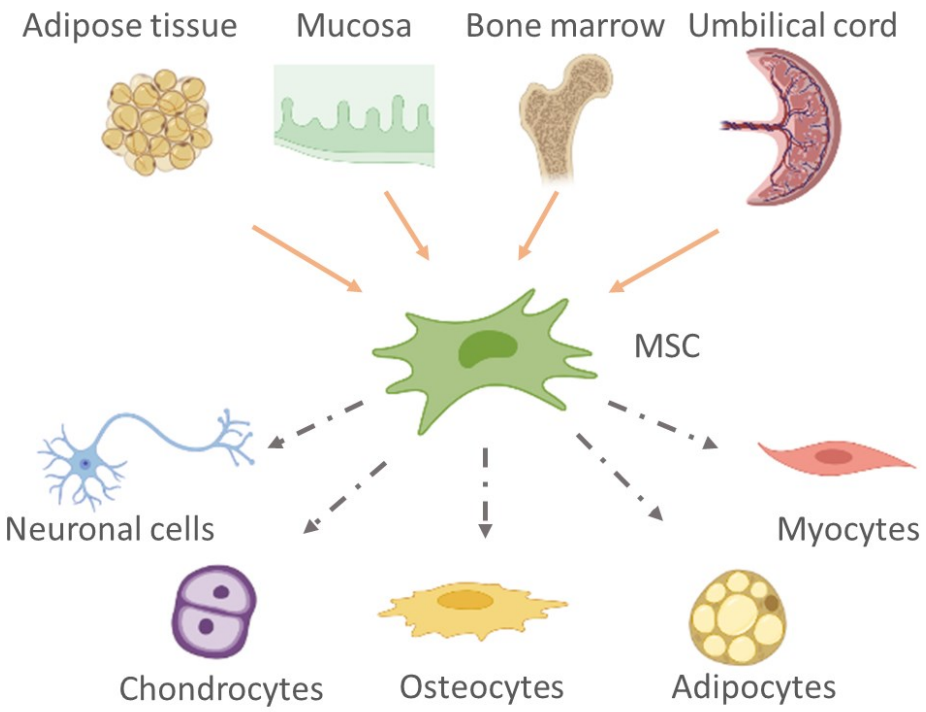

Figure 1: The common sources of human MSCs (solid arrows) used for therapy and the mesodermal lineages that MSCs are capable to differentiate (dashed arrows). Created with BioRender.com.

Finally, in 2006, the International Society for Cellular Therapy made the first attempt to define accurate characteristics of MSCs. These defined characteristics are as follows: 1. MSCs must be self-renewing and adhere to plastic surfaces. 2. MSCs must express surface proteins CD105, CD73, and CD90, while lacking expression of CD45, CD34, CD14 or CD11b, CD79 alpha or CD19, and HLA-DR. 3. MSCs must demonstrate the ability to differentiate into osteoblasts, adipocytes, and chondrocytes in vitro (Dominici et al., 2006). Nonetheless, in recent decades, MSC-like cells have been isolated from nearly all body tissues, including fat, mucosa, skeletal muscle, dental pulp, placenta, amniotic fluid, blood, umbilical cord blood, menstrual blood, lung, and liver, **(Figure 1)**. The main similarity among these cells lies in the expression of cell-surface markers similar to those of bone marrow-derived MSCs and their potential to differentiate at least into bone, fat, and cartilage (Gopalarethinam et al., 2023).

2.3 MSC in Immunity

In recent decades, the significance of mesenchymal stromal cells (MSCs) as an obligatory layer of the immune system has gained considerable recognition. In addition to their stem cell-like differentiation capacity, MSCs possess broad immunoregulatory properties that facilitate interplay between MSCs and both the innate and adaptive immune systems (Brandau et al., 2010;Jakob et al., 2010;English, 2013). Studies have highlighted the functional similarities between MSCs and innate immune cells, as MSCs exhibit cytokine secretion,

immunomodulatory mediator release, chemokine-regulated migration, and the expression of surface molecules for interaction with different leukocyte subsets (Brandau et al., 2010).

Broadly, MSCs' ability to exert their immunomodulatory effects through direct cell-cell contact as well as paracrine mechanisms has been demonstrated (Singer and Caplan, 2011;Dumitru et al., 2014). They are known to downregulate pro-inflammatory functions while enhancing the effector functions of other immune cells (Petri et al., 2017). Specifically, MSCs have demonstrated a prolonging effect on immune cell survival in vitro (Petri et al., 2017). In rodent models, MSCs cocultured with dendritic cells (DCs) upregulate Galactin-1 (Gal-1), leading to the induction of a tolerant immunophenotype in DCs. In an experimental autoimmune uveitis model, MSCs exhibited the capability to inhibit DC maturation (Jiang et al., 2005). Furthermore, human adipose-derived MSCs have shown similar effects on B-cells by reducing antigen production. Notably, under inflammatory conditions, MSCs exhibit an enhanced suppressive capacity against B cells by promoting the induction of regulatory B cells (Bregs) and inhibiting B cell proliferation (Qin et al., 2015).

After injury or during an infection, an immune response is mounted to provide protection against pathogens. Once the invading pathogen has been eliminated, the role of mesenchymal stem cells (MSCs) in the inflammatory phase is primarily to resolve inflammation and initiate regenerative phase: **1.** MSCs exposed to pro-inflammatory cytokines such as IFN-γ, TNF-α, IL-1α, and IL-1β readily respond to counter these pro-inflammatory mediators. This activation or licensing of MSCs leads to the release of high amounts of chemokines and immunosuppressive factors (English et al., 2007;Hemeda et al., 2010;Yu et al., 2019). **2.** The CXCR3 and CCR5 ligands are thought to attract inflammatory immune cells into close proximity with MSCs. As a result, the immunosuppressive factors exert amplified effects on immune cells due to their highest concentration at the source. MSC-derived indoleamine 2,3-dioxygenase (IDO) depletes tryptophan in the local area, inhibiting lymphocyte proliferation. Additionally, factors such as TGF-β, IL-6, IL-10, TSG6, LIF and PGE2 contribute to the suppression of both adaptive and innate inflammatory activity (Bernardo and Fibbe, 2013;English, 2013).

Table 1: Immune and trophic regulation mediated by MSCs.

MSC factor	**Target cell**	**Effect**
Ang-1	Endothelial cells/ Pericytes	Angiogenesis
B7-H4	T Cells	Immunosuppressive
BDNF	Neurones/ Glial cells	Neuroprotective
bFGF	Endothelial cells/ Stem cells	Angiogenesis/ Stemness
CCL- 2, 3, 4, 5, 6, 20	Immune cells/ MSCs	Chemoattraction/ Immune & MSC activation
CXCL- 2, 3, 5, 10, 11, 12	Immune cells/ MSCs	Chemoattraction/ Immune & Stromal cell activation
Galectin-1	T Cells	Immunosuppressive
G-CSF	Granulocytes/ Hematopoietic stem cells	Promotes survival/ Mobilisation/ Hematopoiesis
GDNF	Neurones/ Glial cells	Neuroprotective
GM-CSF	Immune cells (T cells, Macrophages, Granulocytes)	Immune activation
HGF	Endothelial cells/ Epithelial cells/ T cells/ Stem cells	Development/ Myogenesis/ Wound healing
HLA-G	T/ NK cells	Immunosuppressive
HO-1	Immune cells/ MSCs	Immunosuppressive
IDO	T cells/ Dendritic cells	Immunosuppressive
IGF	Neurones/ Muscle/ Cartilage/ Bone	Trophic factor
IL-10	Macrophages/ T cells/ Immune cells	Th2 response/ Immunosuppressive
IL-6	T cells/ Monocytes	Anti or Pro - Inflammatory (context dependent)
IL-8	Immune cells/ MSCs	Chemoattraction/ Angiogenesis
KGF	Epithelium/ Keratinocytes	Wound healing
LIF	T cells	Inhibits Proliferation
M-CSF	Monocytes/ Macrophages	Differentiation/Survival/ Proliferation

MIF	Immune cells/ MSCs	Chemoattraction/ Inflammation/ Wound healing
MMP- 2, 9	Neutrophils/ Stroma	Regulation/ Angiogenesis/ Wound healing
NGF	Neurones/ T cells	Survival/ Growth/ Maturation
PDGF	Fibroblasts/ Smooth muscle/ Glial cells	Angiogenesis/ Proliferation
PD-L1, PD-L2	T cells	Immunosuppressive
PGE2	T cells	Immunosuppressive
Semaphorin-3A	Neurones	Regulation of neural development
TGF-β	Immune cells/ MSCs	Immunosuppressive/ Differentiation/ Growth/ Apoptosis
TIMP- 1, 2	Inhibitor of MMPs	Extracellular matrix remodelling
TSG-6	Immune cells	Migration/ Extracellular matrix stability
Type 1 IFN	Immune cells	Proinflammatory
VEGF	Endothelial/ MSCs	Angiogenesis

Furthermore, MSCs play a crucial role in transitioning from the inflammatory phase to the proliferation phase. This progression is a critical step, as failure to proceed can lead to chronic inflammation and chronic wounds. The simultaneous potent suppression of immune cells mediated by MSCs represent important features of MSCs as immune regulators. These characteristics have consequently led to their therapeutic use in autoimmune and inflammatory diseases (English, 2013;Galderisi et al., 2022).

2.3.1 MSCs in Neutrophil Immunoregulation

Neutrophils are phagocytotic granulocytes, crucial for host innate defence against pathogenic microorganism. Free floating cells and tissue resident cells, like activated macrophages, recruit and activate neutrophils by chemoattractants like IL-8, CXCL1, CXCL2, CXCL5, CCL3 and CCL4. Being the first cells to arrive at site of inflammation, neutrophils play an important role in defend against pathogenic microorganism. Neutrophils detect microbial components thru damage-associated molecular patterns (DAMPs) detected the microbes are phagocytized and digested by fusion with the phagosome containing acidic hydrolases and microbicidal polypeptides and proteins (Amulic et al., 2012;Foote et al., 2017). A critical phase in the acute

immune response of neutrophils occurs if no microbial enemy is encountered. This will lead to a release of their granules into extracellular space and can cause severe damage to the surrounding tissue, specifically if combined with the release of reactive oxygen species (oxidative burst). In recent studies, it could be shown that MSCs exhibit a broad spectrum of immunoregulatory factors associated with recruitment and activation of neutrophils and suggest a complex functional interaction between tissue-resident MSCs and neutrophils. MSC are affecting neutrophils recruitment and evasion into the side of inflammation in an interleukin-8 (IL-8) and macrophage migration inhibitory factor (MIF) manner (Brandau et al., 2010). Upon TL3-activation, MSC are releasing anti-inflammatory type I interferons (IFN-I) suggesting an important role for MSCs in the early phase of inflammation. Once challenged, MSCs are sensitized to a subsequent microbial encounter through an autocrine induced amplification of TLR-3 which activation resulting in an enhanced inflammatory cytokine (IL-6, IL-8) production. The stimulation of MSC via TLR3 activates p38-MAPK and NF-kB inducing the release of IL-6, IL-8 and IFN-I while the autocrine priming is suggested to be mediated by IFN-β or inhibiting the production of reactive oxygen species (ROS) regulation exuberant neutrophil cytotoxicity (Dumitru et al., 2012;Dumitru et al., 2014;Jacobs et al., 2014).

2.3.2 MSCs in T Cell Immunoregulation

T cells play a crucial role in adaptive immunity, orchestrating immune responses against pathogens and regulating immune homeostasis. The balance between T cell activation and suppression is essential for maintaining immune equilibrium (Bonilla and Oettgen, 2010). Mesenchymal stromal cells (MSCs), known for their multipotent differentiation capacity and immunomodulatory properties, have emerged as key regulators of T cell immunity (English, 2013). MSCs have gained increasing attention as potent regulators of T cell immunity. Recent studies have shed light on the immunoregulatory mechanisms MSCs modulate T cell function, complementing their role alongside thymic epithelial cells in the T cell selection process (Bernardo and Fibbe, 2013;Chen and Flies, 2013). Notably, the interaction between MSCs and T cells have revealed intriguing findings. Surprisingly, Tse et al. demonstrated a suppressive effect of MSCs on the proliferation of allogeneic peripheral blood mononuclear cells (PBMCs) and T cells in vitro (Tse et al., 2003). Subsequent studies have further elucidated the suppressive effects of MSCs on T cells, particularly in the context of blocking T cell activation and differentiation into Th1 and Th17 cells when cocultured with activated $CD4^+$ cells. These suppressive effects were associated with an upregulation of interleukin-10 (IL-10) and an increase in the percentage of regulatory T cells (Tregs) (Di Nicola et al., 2002;Soleymaninejadian et al., 2012). In addition to their ability to suppress

T cell proliferation, MSCs have been shown to effectively inhibit mitogen-induced T cell proliferation and induce Tregs across different tissue origins (Di Nicola et al., 2002). These findings provide evidence for the broad immunomodulatory capacity of MSCs in regulating T cell responses.

2.4. Exosomes

Exosomes are a subset of extracellular vesicles (EVs) that play a major role in intercellular communication and the exchange of information within the body. EVs, which include exosomes, encompass a highly heterogeneous group of cell-secreted vesicles composed of lipid bilayers. Initially characterized as waste particles involved in the elimination of cellular components, exosomes are now recognized as vital mediators of paracrine signalling, facilitating the transfer of cargo such as lipids, proteins, and RNA/DNA between cells and tissues throughout the organism. Exosomes are a subtype of extracellular vesicles that typically range in size from 30 to 150 nm and are defined by common exosomal markers CD9, CD63 and CD81 (Lotvall et al., 2014;Théry et al., 2018).

EVs are generated through the endosomal pathway within the cell. As intraluminal vesicles within multivesicular bodies (MVBs), exosomes are formed through inward budding of the limiting membrane of MVBs, resulting in packaging of specific cellular components into these vesicles. Once released into the extracellular environment, exosomes can traverse various body fluids, including blood, urine, and cerebrospinal fluid, enabling their cargo to reach distant target cells and tissues. The composition of exosomes, including their proteins, lipids, and nucleic acids, reflects their cellular origin and can be selectively packaged and delivered to recipient cells, allowing for the transfer of signalling molecules and genetic information (Colombo et al., 2014;Kalluri and LeBleu, 2020).

Exosomes have emerged as key players in multiple physiological and pathological processes, demonstrating their diverse functions in intercellular communication. They participate in immune modulation, angiogenesis, tissue regeneration, and the progression of various diseases, including cancer, neurodegenerative disorders, and cardiovascular diseases. Through the transfer of bioactive molecules, exosomes can influence cellular behaviour, mediate immune responses, and modulate the microenvironment of target cells (Kalluri and LeBleu, 2020).

2.6 MSCs in Therapy

Clinical trials of MSC therapies have demonstrated the potential therapeutic benefits in various diseases, leading to regulatory approvals in different countries **(Figure 2)**, (Jovic et al., 2022). However, several challenges persist, including MSC immunogenicity and decreased viability

in vivo (Barrachina et al., 2017), and the risk of rejection with repeated infusions (Skrahin et al., 2014;Joswig et al., 2017). These issues may contribute to the limited efficacy reported in many trials. Understanding the molecular mechanisms underlying these challenges is vital for improving MSC product manufacture.

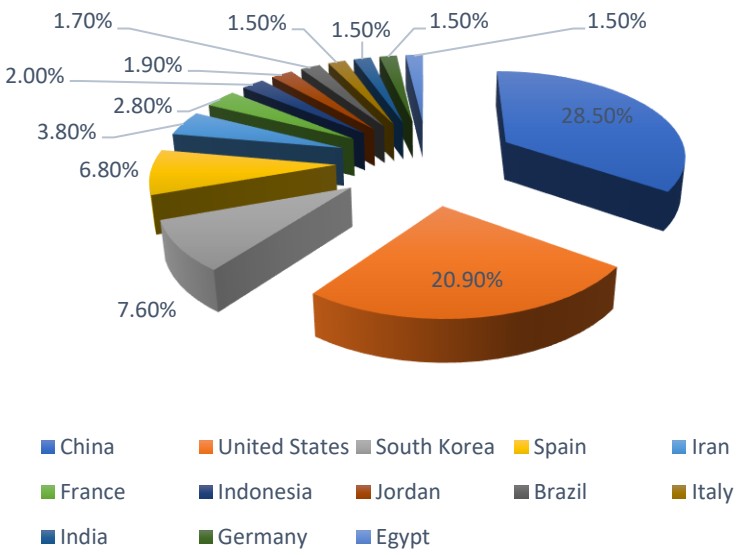

Figure 2: International percentual distribution of interventional and observational clinical trials based on 2021 (cut off:1,5%).

The biggest challenge in safety and success for clinical application of MSC remains in their donor heterogeneity (Joswig et al., 2017;Wilson et al., 2019). The substantial heterogeneity further assumes among MSCs from different tissues (Barrett et al., 2019;Kanazawa et al., 2021;Zhou et al., 2021) and may amplify by culture conditions altering the activation status of MSCs **(Figure 3)**. Overcoming this heterogeneity, influenced by these factors is vital (Alt et al., 2012;Siegel et al., 2013). For this, fully characterizing the biological function and biomarkers of MSCs and defining appropriate stimulations to polarize MSCs' immunoregulatory function is crucial for their effective clinical application and part of this cumulative work. A concerning development, in countries missing medical and safety regulations for MSC therapies, clinics offering unconfirmed exogenous stem cell therapies and starting to threaten the development of MSC therapies (Dimmeler et al., 2014; Rubin, 2018).

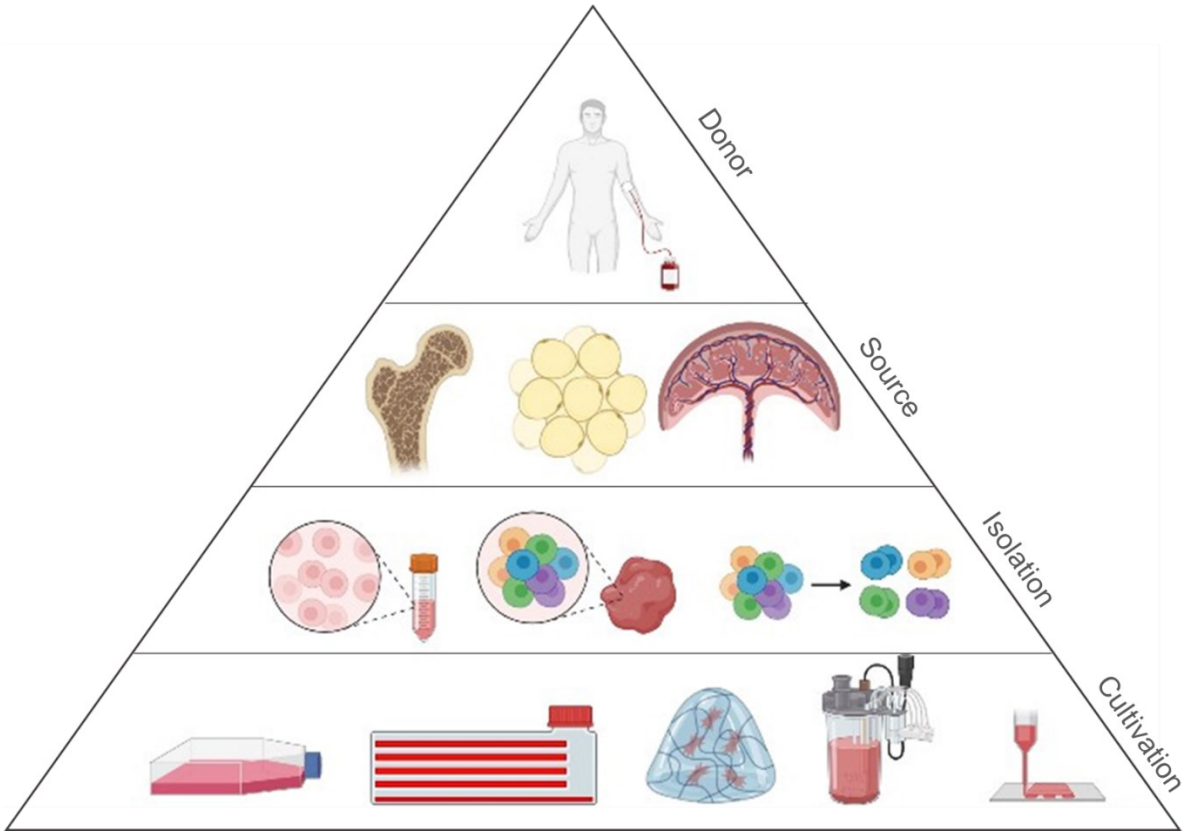

Figure 3: MSCs heterogeneity at multiple levels. Factors influencing MSC heterogeneity include, vertically: Donor characteristics, tissue source, cell isolation techniques, cell culture environment, altered (Zhou et al., 2021). Created with BioRender.com.

2.7 MSC-EV Therapies

Initially, clinical models have shown that MSCs are capable of migrating to areas of tissue damage, while recent studies suggest that MSCs are not able to reach the site of tissue damage and rather accumulate in the lung and spleen (Schrepfer et al., 2007;Duijvestein et al., 2011;Zhu et al., 2013). Accordingly, evidence accumulates that immunoregulation by MSCs is associated with paracrine mechanisms and extracellular vesicles (EVs) are suggested as potential mediators of therapeutic effects (Blazquez et al., 2014;Kordelas et al., 2014;Monsel et al., 2016). EVs contain a multitude of bio- and immunoactive molecules such as cytokines, enzymes, nucleic acids and other proteins which are resembling a comparable protein spectrum of their cell of origin (Fierabracci et al., 2015;Théry et al., 2018). Synoptically, MSC-EVs exert comparable therapeutic effects like MSCs (Kordelas et al., 2014;Wang et al., 2016;Álvarez et al., 2018). Thereby, MSCs effectiveness is more demonstrated by paracrine signalling, while nesting in the lung for example, and less by their ability to migrate into diseased tissue and local differentiation (Gupta et al., 2021;Liu et al., 2021;Miceli and Bertani, 2022). Further, it is evident that EVs properties, cargo and functions resemble the MSC population they derived, in

its physiological and activation state. MSC-EVs are therefore employed in similar disease settings as MSCs (Cai et al., 2020). Additionally, MSC-EVs represent an easy to handle, store and sterile therapeutic tool, whose application minimizing risks for patients compared to conventional MSC cell based therapy (Kordelas et al., 2014;Börger et al., 2017; Matsuzaka and Yashiro, 2022).

The anti-inflammatory effects in MSC-EV therapies are accompanied by regenerative mechanism. In an in vitro model of spinal cord injury MSC-EVs were able decrease apoptosis and inflammation by inhibition of phosphatase and tensin homolog (PTEN) and nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB), (Zhang et al., 2021). In Preclinical models of Patellar tendon injury, administered EVs lead to an increase of anti-inflammatory cytokines (IL-10, IL-4) and simultaneous healing benefits of tendons by increasing the local abundance of progenitor cells (Shi et al., 2019). The most prominent limitations, MSC and MSC-EV remains the reducibility of clinical benefit by the obligatory use of different MSC donors or different batches or cultivation methods of MSCs. Taking into account that EVs are not able to adept in their function, once they are delivered in the extracellular space neither are able to adapt in their function, while their function is donor dependent or can by reversed by the smallest differences while cultivating (Cai et al., 2020;Madel et al., 2023). It is obvious that mechanisms are needed to define or prime MSC-EVs to determine their function. Induced hypoxia of MSCs has shown to be a sufficient priming method to generate EVs promoting graft survival and neovascularization in a fat grafting. In particular priming with pro-inflammatory cytokines of MSC resulting in an anti-inflammatory response of MSC, and primed MSC derived EVs are of particular interest for therapeutic approaches. IFN-y priming of MSC has shown to decrease inflammatory and fibrotic markers resulting in improved therapeutic efficiency in a systemic sclerosis mice model (Rozier et al., 2021). Interestingly, the effect of IFN-γ and hypoxia priming together is questioned to be efficient (Peltzer et al., 2020). In the subsequent studies, we aim to elucidate the distinctions in both response and pathways implicated in cytokine priming of MSCs. Additionally, we will proceed to assess their immunoregulatory and therapeutic potential. Particularly, these investigations take place within the context of a GvHD model.

2.7.1 MSC-EV in GvHD Therapy

In T-lymphocyte regulation and activation, the importance of PD-1 receptor is evident. This is strikingly demonstrated by the increased vulnerability to autoimmune diseases observed in PD-1 knockout mice (Nishimura et al., 1998;Salama et al., 2003). The involvement of the

PD-1 pathway in the immunoregulation of T cells strongly implies its pivotal role within murine models of GvHD (Tobin et al., 2013;Fujiwara et al., 2014;Davies et al., 2017). The expression of PD-L1 has been identified on non-hematopoietic cells, including MSCs, as well as on hematopoietic cells. Meanwhile, PD-1 ligand 2 (PD-L2) is primarily associated with antigen-presenting cells (APCs), though reports also indicate its presence in MSCs (Yamazaki et al., 2002;Chinnadurai et al., 2014b;Chen et al., 2018). Previous studies have indicated the occurrence of PD-L1 within microvesicles, extracellular vesicles, and as independent entities or membrane-bound particles within the MSCs' secretome (Goncalves et al., 2017;Chen et al., 2018).

3. Aim of the Study

This study aims to augment our understanding of MSCs role in immunity, focusing on their immune response within a pro-inflammatory environment, as well as their subsequent intercellular communication with other immune cells. MSCs are not only progenitor cells with the potential to differentiate into diverse tissue cell types but also possess ability to localize to damaged and inflamed tissues, where they exhibit profound immunomodulatory and regenerative functions. Recognizing that these inflammatory environments can potentially influence the immunomodulatory functions of MSCs, we chose to investigate the effects of representative inflammatory cytokines such as IFN-γ, TNF-α, and IL-1β. In the first part of our study, we decipher the cell biological and kinetic responses of MSCs to these cytokines. Our goal is to understand the mechanisms through which cytokine signals are transformed into immunoregulation by MSCs. In the second study, we employ this knowledge in a therapeutic context of systemic inflammation, focusing on a Graft-versus-Host Disease (GVHD) model. By strategically applying our findings, we elucidate the capabilities of primed MSCs, their EVs compared to their unstimulated complements in modulating alloreactive T cells. The gained insights can provide new implications to potentiate the applicability and efficiency of cellular or EV-based therapeutics.

4. Research Questions

What alterations occur in the immune responses, cytokine secretion, and surface protein expression of MSCs due to multi-cytokine priming?

How does priming influence MSCs' intracellular pathways and autocrine modulation? Are responses consistent across MSCs from different tissues?

What effects does multi-cytokine priming have on the intercellular communication between MSCs and other cells, such as PMNs and T lymphocytes?

What are the prospective clinical advantages of applying cytokine primed MSCs or their EVs in interventions targeting Graft-versus-Host Disease?

5. Direct Citation of the Cumulative First Author Publications

5.1 TNF-alpha and IL-1beta sensitize MSC for IFN-gamma signalling and enhance neutrophil recruitment

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Running title:

Multi-cytokine stimulation augments the pro-inflammatory activity of tissue-resident MSC

Keywords:

Mesenchymal stromal cells, neutrophil recruitment, IL-1β, IL-8, TNF-α, IFN-γ, STAT-5, p38-MAPK

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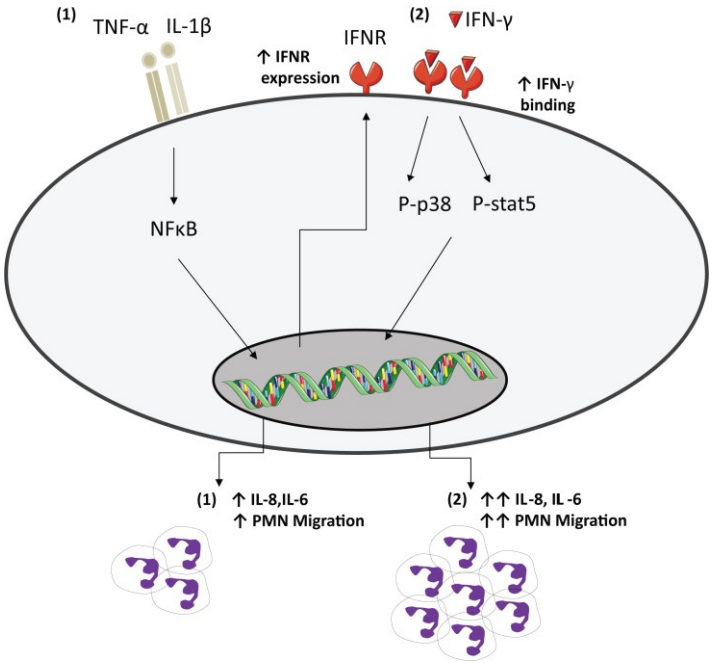
Abstract:

During inflammatory processes, tissue environmental cues are influencing the immunoregulatory properties of tissue-resident mesenchymal stem/stromal cells (MSC). In this study, we elucidated one of the molecular and cellular responses of MSC exposed to combinations of inflammatory cytokines. We showed that during multi-cytokine priming by tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β) and interferon-gamma (IFN-γ), IL-1β further augmented the well-established immunoregulatory activity induced by TNF-α/IFN-γ. On the molecular level, TNF-α and IL-1β enhanced the expression of interferon-gamma receptor (IFN-γR) via nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NF-κB) signalling. In turn, enhanced responsiveness to IFN-γ stimulation activated STAT5 and p38-MAPK signalling. This molecular feedback resulted in an increased IL-8 release and augmented recruitment of polymorphonuclear granulocytes (PMN). Our study suggests the possibility that responses of MSC to multi-cytokine priming regimens may be exploited therapeutically to fine-tune inflammatory activity in tissues.

Graphical Abstract

TNF-α, IL-1β and IFN-γ activate MSC to secrete IL-8 and interleukin-6 (IL-6). IL-8 release enhances PMN recruitment. During phase 1 the pre-priming by TNFα and IL-1β induces IFN-γR expression (1). Binding of IFN-γ to the IFN-γR causes a sequential induction of p38-MAPK and STAT5 phosphorylation followed by increased IL-8 secretion and consequently enhanced PMN recruitment (2).



Introduction

Mesenchymal stromal cells (MSC) are non-hematopoietic, fibroblast-like progenitor cells, capable of differentiating into different lineages, such as chondrocytes, osteoblasts and adipocytes. They are defined by their plastic adherence, the expression of specific MSC surface markers and the absence of certain endothelial and hematopoietic cell surface antigens (Horwitz et al., 2005;Dominici et al., 2006a;Luz-Crawford et al., 2016). Originally identified in the bone marrow, they have been isolated from many peripheral tissues or body fluids, such as adipose tissue, connective tissue, umbilical cord, menstrual blood and nasal mucosa (Musina et al., 2005;Jakob et al., 2010;Gregoire-Gauthier et al., 2012;Kim et al., 2017).

Next to their stem cell-like properties, MSC also have immunoregulatory activity. During inflammatory processes, these immunoregulatory properties of tissue-resident MSC are influenced and controlled by their local tissue environment, which enables them to readily respond to pathogenic and cytokine stimuli (Delarosa et al., 2012; Bernardo and Fibbe, 2013;Barrachina et al., 2016;Petri et al., 2017). MSC exert their

immunomodulatory properties via both, direct cell-cell-contact and paracrine mechanisms. MSC display multidirectional immunomodulatory properties and are known to downregulate pro-inflammatory functions of dendritic cells (Zhang et al., 2004), T-cells and B-cells (Luz-Crawford et al., 2013;Franquesa et al., 2015b;Zhang et al., 2017). MSC can also polarize NK cells to a pro-inflammatory or an anti-inflammatory phenotype (Petri et al., 2017). One of the first tasks of MSC during acute inflammation may be the recruitment and activation of neutrophils (Brandau et al., 2010;Cassatella et al., 2011;Brandau et al., 2014). Neutrophils arrive at the site of inflammation within minutes and subsequently regulate the recruitment and function of other immune cells such as monocytes, dendritic cells and lymphocytes (Silva and Correia-Neves, 2012;Kienle and Lammermann, 2016). During early phases of inflammation, pro-inflammatory cytokines or pathogens activate MSC immunoregulatory function, which may lead to the increased recruitment of neutrophils to the site of inflammation (Brandau et al., 2010;Dumitru et al., 2014). Subsequently, MSC may inhibit the apoptosis of neutrophils and downregulate the intensity of oxidative burst via IL-6 (Raffaghello et al., 2008; Brandau et al., 2010).

Under inflammatory conditions MSC are exposed to various combinations of cytokines. MSC exposed to TNF-α and IFN-γ upregulate a variety of immunoregulatory proteins, all of which are able to regulate innate and adaptive immunity. The combined activity of TNF-α and IFN-γ can, for example, enhance the expression of immunoregulatory proteins such as PD-L1, PD-L2, IL-8, IL-6, TGF-β, IDO, PGE_2 and adhesion molecules (English et al., 2007; Ren et al., 2008;Dumitru et al., 2014;Barrachina et al., 2016;Petri et al., 2017). TNF-α often activates signalling via NF-κB (Cheng et al., 2019). Exposure to IFN-γ primarily leads to a phosphorylation of STAT1 and STAT3 in MSC resulting in a suppression of T cell proliferation among other immunoregulatory functions (Vigo et al., 2017). Like TNF-α, also IL-1β results in comparable changes of MSC' immunoregulatory function mainly via NF-κB signalling (Huh et al., 2014a;Huang and Chen, 2017). Thus, it is well established that cytokines can modulate the immunoregulatory activity of MSC (Apetoh et al., 2007;Hemeda et al., 2010; Gebler et al., 2012). However, it is important to note that the response of MSC to complex cytokine compositions in a pro-inflammatory environment remains poorly characterized, since the majority of published studies focused on simpler single or double cytokine stimulations. In this study, we obtained further insight into the molecular mechanism elicited in MSC in response to multi-cytokine stimulation. We uncovered a regulatory network, in which inflammatory cytokines augment the response of MSC to IFN-γ with consequences for IL-8 secretion and subsequent neutrophil recruitment.

Results

Multifactorial priming modulates the cytokine secretome of MSC

Priming or licensing by cytokines is known to modulate immunoregulatory properties of MSC (Apetoh et al., 2007;Hemeda et al., 2010;Gebler et al., 2012). In order to explore the potential response of MSC in diverse cellular environments, we first tested several cytokine combinations, including the well-established combined stimulation by IFN-γ and TNF-α (Hemeda et al., 2010;Jakob et al., 2010;Gebler et al., 2012), but also included IL-2, a cytokine primarily produced by activated T cells and IL-1β, a cytokine primarily secreted by activated macrophages. Based on previous studies (Hemeda et al., 2010;Jakob et al., 2010; Dumitru et al., 2014;Petri et al., 2017), the read-out MSC cytokine secretion panel was designed with a focus on the potential of MSC to modulate the biology of PMN (Dumitru et al., 2012; Petri et al., 2017). As indicated in figure 1a, single cytokine priming induces a moderate to strong secretion of selected cytokines when compared to unstimulated controls. When combined cytokine stimulation was used, we observed a broader induction of the response, in particular for a low concentration of IL-1β (10ng/mL) in combination with IFN-γ and TNF-α. Because we were primarily interested in the MSC/PMN interaction, we chose IL-8, a key cytokine in neutrophil activation and recruitment, for further analysis of the MSC response. When we repeated initial experiments with additional donors, we observed a stronger induction of IL-8, when IL-1β was added to the IFN-γ/TNF-α combination (Fig. 1B). This induction of IL-8 increased over time. In addition, triple cytokine stimulation of MSC resulted in a very strong induction of neutrophil migration towards conditioned MSC medium (Fig. 1C). Application of inhibitory antibodies revealed that this induction of neutrophil migration was mediated by IL-8, with little or no contribution of IL-6 (Fig. 1C).

Multifactorial priming by TNF-α/IFN-γ/IL-1β activates distinct signalling pathways in MSC

In the next set of studies, we wanted to analyse signalling pathways involved in triple cytokine stimulation. We compared triple cytokine stimulation to unstimulated control MSC and to the stimulation by the fairly well characterized licensing of MSC via IFN-γ and TNF-α combination. In the early time points, 2h and 4h post stimulation, no strong differences in NF-κB/p65 translocation to the nucleus (NUC) were observed when comparing double with triple cytokine stimulation (Fig. 2A and B). In addition, no differences of p50/p52 translocation

and phosphorylation of STAT3 and STAT5 were observed at these early time points (data not shown). Nevertheless, both double and triple combination, induced NF-κB translocation as compared to unstimulated MSC. We next analysed later time points of stimulation that is 6h and 12h. In contrast to earlier time points, TNF-α/IFN-γ/IL-1β now upregulated phosphorylation of p38-MAPK, total STAT5 protein and phospho-STAT5 (Fig. 2C-G). Of note, at the 6 hour time point, the triple cytokine stimulation transiently induced phospho-p38 and phospho-STAT5 over levels observed for the IFN-γ/TNF-α combination (Fig 2E, G). Interestingly, these later time points also revealed a substantial upregulation of total STAT5 protein particularly by TNF-α/IFN-γ/IL-1β (Fig 2F). These data show the enhanced activation of distinct signalling pathways by the triple cytokine stimulation.

Next, we tested the biological relevance on MSC-PMN crosstalk of the respective signalling pathways by using specific inhibitors and IL-8 secretion as a read-out. NF-κB signalling was directly inhibited together with the onset of multi-cytokine priming to block the early signalling response. Based on the induced activation of p38-MAPK and STAT5 phosphorylation at later time points (Fig. 2), we titrated the respective inhibitors for these pathways 4h after initiation of cytokine priming. Both canonical NF-κB inhibitors, the direct p65 signalling inhibition (NF-κB Activation Inhibitor II) and inhibition of TNF-α stimulated NF-κB activation (NF-κB Activation Inhibitor III), decreased IL-8 secretion after TNF-α/IFN-γ/IL-1β priming (Fig. 3). Furthermore, delayed inhibition of p38-MAPK and STAT5 inhibition also reduced IL-8 secretion (Fig. 3). These results suggest prolonged and sustained cytokine signalling in MSC exposed to the triple cytokine combination.

TNF-α and IL-1β stimulate MSC to express interferon-gamma receptor

We next considered the possibility that the cytokines affect the expression of their own receptor or of "partner" cytokine receptors. To test this we compared the expression of IFN-γR, IL-1R1 and TNFR after double and triple cytokine priming. Interestingly, the triple cytokine stimulation substantially upregulated IFN-γR and IL1R over stimulation with IFN-γ/TNF-α combination (Fig. 4A). In contrast, the triple cytokine stimulus only slightly induced the expression of TNFR. We next uncoupled the three cytokines from each other and tested sequential activation of MSC by two cytokines followed by a second single cytokine stimulation. In line with the receptor expression (Fig 4A), from all combinations tested, the sequential activation of MSC by IL-1β/IFN-γ followed by TNF-α resulted in the lowest secretion of IL-8, nevertheless still showing a clear induction over the unstimulated control. (Fig. 4B). Finally, we considered the possibility that the effect of triple cytokine stimulation is,

at least in part, mediated via regulation of the IFN-γR by IL-1β/TNF-α. Experiments indeed showed that priming by IL-1β/TNF-α upregulated IFN-γR expression (Fig. 4C).

Priming by TNF-α and IL-1β sensitizes MSC for subsequent IFN-γ signalling

In a final set of experiments, we wanted to further dissect and substantiate the mechanism underlying the priming of MSC by TNF-α/IL-1β for a subsequent IFN-γ response. We consider this to be a particular relevant scenario, as TNF-α/IL-1β are produced by activated macrophages in early phases of inflammation, while IFN-γ is produced by recruited T cells at later stages. To address this scenario, we first stimulated MSC by TNF-α/IL-1β in the presence of a NF-κB III activation inhibitor and found that the induction of the IFN-γR by these two cytokines is at least in part dependent on NF-κB (Fig. 5A). In contrast, blockade of STAT5 and p38-MAPK signalling did not affect upregulation of IFN-γR by IL-1β/TNF-α (Fig. 5A). Next, we first stimulated MSC with TNF-α/IL-1β and then blocked STAT5 and/or p38-MAPK signalling during the subsequent IFN-γ stimulation phase. The p38-MAPK inhibitor strongly reduced IL-8 secretion, while STAT5 inhibition showed a moderate reduction of IL-8 secretion in this experimental set up. Of note, the combined use of both inhibitors completely abrogated the induction of IL-8 by IFN-γ stimulation (Fig. 5 B). Consistent with this finding, the capacity of the MSC SN to induce PMN migration was also blocked and reduced to IL-1β/TNF-α control conditions (Fig. 5C). In the last part of the study, we tested effects of MSC priming on PMN functional activity. PMN exposed to triple cytokine primed MSCs showed a substantially improved survival (Fig. 5D), a moderately (not statistically significant) augmented production of ROS in response to bacterial challenge (Fig. 5E) and strongly enhanced production of CCL4 (Fig. 5F). These data show that multi-cytokine stimulation involves a two-step process. The process consists of MSC priming and IFN-γR upregulation/sensitization by TNF-α/IL-1β, which is then followed by an augmented p38-MAPK>STAT5-mediated response to IFN-γ (cf. graphical abstract).

Discussion

Previous studies have shown that pro-inflammatory stimuli, such as bacterial endotoxin, virus fragments or cytokines activate MSC to secrete molecules that regulate innate and adaptive immune cells (Cassatella et al., 2011;Gebler et al., 2012;Dumitru et al., 2014b;Petri et al., 2017). As such, MSC can function as early sensors of inflammatory events and regulate the first immune response by the recruitment of PMN and by regulating PMN immunity itself. Interestingly, exposure to inflammatory or pathogenic signals can sensitize MSC to a subsequent challenge, and thus amplify the immunological activity of MSC (Brandau et al., 2014;Dumitru et al., 2014).

The aim of this study was to elucidate the molecular and immunobiological response of MSC exposed to multi-cytokine priming. Our research question is based on the concept that MSC are not per se immunoregulatory but rather require priming mediators to fully activate this function (English et al., 2007;English, 2013;Dumitru et al., 2014;Petri et al., 2017). To this end, we have tested different combinations and concentrations of pro-inflammatory cytokines to license MSC. Among the tested pro-inflammatory cytokines, the combination of IFN-γ (1000 U), TNF-α (1000 U) and IL-1β (10ng/ml) had the most prominent effect on MSC immune biology. In our model, IL-1β seems to have a "fine tuning" effect, enhancing the already strong licensing effect by TNF-α/IFN-y. However, in this context, we wish to point out that also higher concentrations of IL-1β had a similar effect, albeit less pronounced. We do not have a molecular mechanism at hand to explain this concentration-dependent phenomenon, but realize that the amounts of IL-1β that were used in our study are in line with previous published work utilizing similar concentrations (Huh et al., 2014;Philipp et al., 2018).

Since we showed in our previous work by Dumitru et al. (Dumitru et al., 2014b) the importance of IL-8 in MSC and PMN interaction, we focused on IL-8 secretion as a main read out for the immune response of MSC after stimulation. IL-8 is a potent chemoattractant, promoting PMN recruitment and activation by binding to CXCR1 and CXCR2 receptors (Brandau et al., 2010;Cassatella et al., 2011;Jakob et al., 2013;Brandau et al., 2014). Interestingly, in our study we demonstrated an IFN-γR-dependent IL-8 secretion by multi-cytokine activated MSC via activation of STAT5 and p38-MAPK signalling pathways. Thus, it is conceivable that STAT5 and p38-MAPK represent two separate pathways both activated by IFN-γR. This is in line with published studies showing that STAT5 and p38-MAPK may be

separately regulated via janus kinase in T-cells and macrophage cell lines (Beyer et al., 2011;Gao et al., 2016).

Our multi-cytokine stimulation by TNF-α/IFN-γ/IL-1β aimed to mimic a potential cytokine milieu in early tissue injury or inflammation. Our results are consistent with earlier studies showing substantial secretion of IL-8, IL-6 and MIF by activated MSC (Brandau et al., 2010; Hemeda et al., 2010;Jakob et al., 2010;Dumitru et al., 2014;Petri et al., 2017). We believe that our findings support the existence of an intensive cross-talk between MSC and macrophages in an early stage of inflammation (Ulivi et al., 2014). During this crosstalk, multi-cytokine activated MSC upregulate the IFN-γR. It is well established that lymphocytes, arriving at later stages at the site of inflammation, contribute to the local cytokine milieu by secreting high amounts of pro-inflammatory cytokines such as IFN-γ. Thus, our findings suggest a scenario in which priming of MSC by inflammatory "early cytokines" enhances their capacity to respond to IFN-γ that is secreted by lymphocytes or NK cells arriving at the site of inflammation at a later time-point (Dumitru et al., 2011;English, 2013;Dumitru et al., 2014;Petri et al., 2017; Vigo et al., 2017). In a subsequent phase of inflammation, recruited PMN may contribute to resolution of inflammation (Petri et al., 2017). At this point, we wish, however, to emphasize that other sequences of multi-cytokine stimulation may yield similar outcomes. Already in this study we observed that MSC primed with IFN/TNF and stimulated with IL-1β likewise produce high amounts of IL-8. Thus, it is tempting to speculate that the augmentation of MSC responses by cytokine priming could be a general feature of these cells.

In addition to cytokines, MSC may also be exposed to pathogens and pathogen-derived molecular structures. Of note, Toll-like-receptor (TLR) stimulation of MSC led to a biphasic cytokine response triggered by an augmented TLR binding response (Dumitru et al., 2014). Furthermore, Petri et al. (Petri et al., 2017) demonstrated a time-dependent response of poly(I:C)-challenged MSC towards NK cells, where, at early time-points, activated MSC enhanced NK cell effector function. Thus, our findings may also have implications for the immune response of MSC in infections.

MSC are intensively used and tested as cellular therapeutics to normalize immune responses in diseases associated with autoreactivity or hyperinflammation (Mougiakakos et al., 2012;Zafranskaya et al., 2013;Maria et al., 2017;Curtis et al., 2018). In these therapeutic settings, at least until today, therapeutic MSCs are mostly cultured and harvested from and under steady-state culture conditions. Our findings now offer the appealing possibility to develop optimized in vitro priming protocols for MSC for tailored function and

therapeutic activity. Future studies are warranted to test this possibility in pre-clinical and clinical settings.

In sum, our findings demonstrate the ability of MSC to sense multifactorial signals during different stages of inflammation and their ability to provide targeted responses (Tondreau et al., 2005;Brandau et al., 2010;Cassatella et al., 2011;Jakob et al., 2013; Brandau et al., 2014). Accordingly, we hypothesize that MSC act as local regulators of immunity in inflammatory events by directing homeostasis and activity of motile immune cells, comparable to MDSC or induced regulatory T cells in the tumour environment (Luz-Crawford et al., 2013;Lang et al., 2018). Considering the abundance of stromal cells in many inflamed tissues, we may even consider MSC as an additional layer of immunity in infection and tissue injury.

At the molecular level, our study defines IL-1β as an important stimulus that further augments the immunoregulatory activity of MSC primed by TNF-α/IFN-γ. These findings underline the capacity of MSC to sense and respond to complex cytokine cascades. We identified molecular mechanisms of MSC in response to multi-cytokine priming and showed the consequences for PMN recruitment and cellular inflammation. Based on our findings, potential molecular targets can be defined to downregulate unwanted hyper-inflammation in tissues.

Materials and methods

Isolation and culture of nasal mucosa MSC (nmMSC)

nmMSC were obtained from the inferior nasal concha of healthy individuals (age 30-70 years) at the Department of Othorhinolaryngology, University Hospital Essen (Essen, Germany). The isolation and culture of nmMSC, evaluation of differentiation potential and routine control for immunophenotypic markers were conducted as described before (Jakob et al., 2010). MSC were cultured in DMEM/RPMI-1640 high glucose (50%/50% v/v), supplemented with 2mM L-Glutamine , 100 IU/ml penicillin, 100 mg/ml streptomycin (all ThermoFischer Scientific, Karlsruhe, Germany) and 10% (v/v) heat-inactivated FCS (Merck/Biochrom, Berlin, Germany). All nmMSC used in experiments were between passage 3-6. The ethics committee of the Medical Faculty of the University of Duisburg-Essen approved all studies; each individual donor submitted an informed written consent.

Stimuli, inhibitors, and antibodies

Stimulation of nmMSC was conducted with the following cytokines: IL-1β, TNF-α (Milteny, Bergisch Gladbach, Germany), IFN-γ (PeproTech, Hamburg, Germany), IL-2 (Novartis, Nürnberg, Germany). Inhibitors used for signalling analysis of nmMSC: NF-kB Activation Inhibitor II, NF-kB Activation Inhibitor III, SB202190 (p38-MAPK-MAPK inhibitor), STAT5 Inhibitor (all Merck, Darmstadt, Germany).

Isolation and culture of neutrophils (PMN)

Diluted peripheral blood of healthy donors (1:1, v/v in phosphate buffered saline [PBS]) was subjected to a Biocoll density gradient separation (Merck, Darmstadt, Germany). The neutrophil fraction was collected in a fresh test tube. Erythrocytes were removed by sedimentation with a solution containing 1% polyvinyl alcohol. The resulting PMN were cultured in RPMI-1640 supplemented as indicated above.

Chemotaxis of PMN

The directed migration (chemotaxis) of PMN was determined by using 3µm cell culture insert together with 24-well plates (Sarstedt, Nümbrecht, Germany). Migrated cells were counted by flow cytometry via 123-counting beads (ThermoFischer scinetific). The number of cells migrating in response to stimulus divided by the number of cells migrated toward unconditioned medium was calculated.

Generation of nmMSC conditioned medium for PMN Assays

nmMSC were stimulated with TNFα/IFNγ, TNFα/IFNγ/IL-1β or left unstimulated for 24h. Cells were washed with PBS, fresh was medium added and SN was collected after additional incubation for 24h. Conditioned medium from 4 different MSC donors were pooled and stored in aliquots at -20°C.

Neutrophil apoptosis and CCL4 production

PMN were cultured in two-fold diluted conditioned medium of nmMSC or standard culture medium for 24 h. SN was collected and stored at -20°C for CCL4 ELISA. Remaining PMN were washed with PBS and stained with BD Pharmingen PE Annexin V Apoptosis Detection Kit I. Analysis was performed using a BD FACS Canto II flow cytometer (all BD Bioscience) and Diva Software 8.0.

Respiratory burst of PMN

The release of reactive oxygen species (ROS, respiratory burst) by PMNs, induced by phagocytosis, was measured by oxidation of dihydrorhodamine-123 (DHR-123, Invitrogen) to fluorescent rhodamine-123. Isolated PMNs were pre-incubated in condition mediums of nmMSC or standard culture medium for 1 h. After washing with PBS, PMNs were incubated in the presence or absence of E. coli (strain JM109) with MOI 2,5, 5,10 and 20 in RPMI-1640 supplemented with 10% autologous serum for 15 minutes at 37°C. Subsequently, DHR-123 (2.5µg/mL) was added for 15 minutes and afterwards incubated on ice for 15 min. Analysis was performed immediately using a BD FACSCanto II flow cytometer and Diva Software 8.0.

Cytokine analysis

Cytokine secretion of MSC was measured in the supernatant of cultured MSC. Cytokine concentration of IL-6, IL-8 and CCL4 was either determined by individual ELISA kits (all BioTechne, Wiesbaden, Germany) or simultaneous analysis of IL-6, IL-8, GM-CSF, MIF and CXCL12 from one sample was performed with Bio-Plex Chemokine Assays based on the Luminex xMAP technology (Bio-Rad Laboratories, München, Germany).

Flow cytometric analysis

MSC surface proteins were stained with antibodies diluted in phosphate-buffered saline (PBS) and 3% human serum (HS). The following antibodies were used: CD54-APC (clone HA58, ThermoFischer scinetific), CD119-PE (clone GIR-94), CD120a-APC (clone W15099A, both Biolegend, Fell, Germany), CD121a-FITC (polyclonal goat IgG, BioTechne). Isotype antibodies were used as controls. Analysis was performed using a FACSCanto II flow cytometer and Diva Software 8.0.

SDS-PAGE and Western blot analysis

For SDS-PAGE 1-3 x 10^6 nmMSC were stimulated as indicated and lysed with Urea buffer containing 25 mM HEPES (pH 7.3), 0.1% SDS, 1% Triton X-100, 10 mM EDTA, 10 mM sodium pyrophosphate, 10 mM NaF, 125 mM NaCl, 1% protease inhibitor cocktail I, 1% protease inhibitor cocktail III, and 10% PhosStop. Cell debris was removed by centrifugation, and the lysates were incubated with SDS sample buffer (pH 6.8) 50 mM Tris, 4% glycerine, 0.8% SDS, 1.6% β-mercaptoethanol and 0.04% bromphenol blue). Samples were further analysed by wet immunoblotting on nitrocellulose membranes (GE Healthcare). For immunodetection the following antibodies were used: anti phospho-STAT1 (Tyr701) (clone D4A7), STAT5 (total protein) (clone D3N2B), phospho-STAT5 (Tyr694) (clone D47E7), p38-MAPK (p38α, β, or γ MAPK protein) (clone D13E1), phospho-p38-MAPK (Thr180/Tyr182) (clone D3F9), β-Actin (clone 13E5), Vinculin (clone E1E9V), NF-κB p65 (clone D14E12) and goat anti rabbit IgG HRP (all Cell Signalling Technology, Danvers, MA, USA).

Statistical analysis

All data are shown as means as centre value and errors bars by SD or SEM as indicated. Data were analysed by ratio paired parametric t-test, paired parametric t-test or by one-way analysis of variance (ANOVA) with Tukey's multiple comparison test.

Acknowledgments

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Conflict of interest statement

The authors do not have any conflicts of interest to report.

Figure Legends

FIGURE 1. MSC primed by TNFα/IFNγ/IL-1β show an enhanced and broader cytokine secretion pattern. (A) MSC from the nasal mucosa of three different donors were independently stimulated with different cytokine combinations: TNFα (1000 U/ml), IFNγ (1000 U/ml), IL-1β and IL-2 at concentrations as indicated (low = 10 ng/ml; high = 100 ng/ml) for 24h. Colour code mean of cytokine concetrations in supernatant analysed by a bioplex assay. Absolute concentrations (pg/ml, mean ± SD of 3 donors) are indicated as white numbers. (B) MSC from three different donors were independently stimulated with TNFα/IFNγ or IFNγ/TNFα and 10ng/ml IL-1β, SN was collected after 6h, 12h and 24h, and IL-8 was measured for each donor by a single ELISA. Statistical difference between TNFα/IFNγ and IFNγ/TNFα/IL-1β data points was calculated (P-values from left to right: **P=.0051; **P=.0021; ***P=.0001). (C) Migration of PMN through 3 µm trans-wells towards SN of TNFα/IFNγ/IL-1β primed MSC in the presence of inhibitory antibodies against IL-6 (1 µg/ml) and IL-8 (1 µg/ml). PMN from three different donors were separately incubated with pooled supernatant harvested from three independent MSC donors (P-values from left to right: ***P=.0004, n.s. P=.9882, **P=.0013, **P=.0027). For all experiments One-way ANOVA with Tukey's multiple comparison test was used to test statistical significance. Data is shown as mean (center value) and error bars indicate SD.

FIGURE 2. Sequential activation of cytokine-induced signaling in MSC. (A, B) Activation of NFkB-I is shown by translocation of p65 (p65) into the nucleus. MSC were stimulated with TNFα/IFNγ or TNFα/IFNγ/IL-1β for 2h, lysed via cell fractionation for cytosol (CYT) and nucleus (NUC) and subjected to western blot analyses. Vinculin was used as loading control of cell lysates. (A) Representative Western Blot analysis from 1 out of 3 experiments performed with independent donors. (B) Densitometric analysis of Western blots by Image Studio Lite. Ratio of cytosolic p65 to nuclear p65 is shown.(C-G) MSC were independently stimulated with TNFα/IFNγ or TNFα/IFNγ/IL-1β, lysed and subjected to western blot analysis after indicated time points with antibodies against phosphorylated STAT1 (p-STAT1), p38 MAPK (p38), phosphorylated p38-MAPK (p-p38), STAT5 (STAT5) and phosphorylated STAT5 (p-STAT5). β-actin was used as loading control of cell lysates. (C) Representative Western Blot analysis from 1 out of 3 experiments (p-STAT1 and β-actin) and 1 out of 4 experiments (p38, p-p38, STAT-5, p-STAT5 and β-actin) performed with cells from independent donors. (D- G) Densitometric analysis of Western blots by Image Studio Lite.

(D, F and G) total protein was normalized to loading control. (E) Phosphorylated p38 was normalized to total p38. (D-G) data is shown as fold-increase over unstimulated MSC. Unstimulated samples were set as one. Ratio paired t-test was used to test statistical significance. All data is shown with mean as the centre value and error bars indicate SD (n= 3-4 experiments). P-values indicated in the figures from left to right: Fig.2D n.s. P=.3035, n.s. P=.8287; Fig.2E *P=.0395, n.s.=.0726; Fig.2G **P=.0068, **P=.0025.

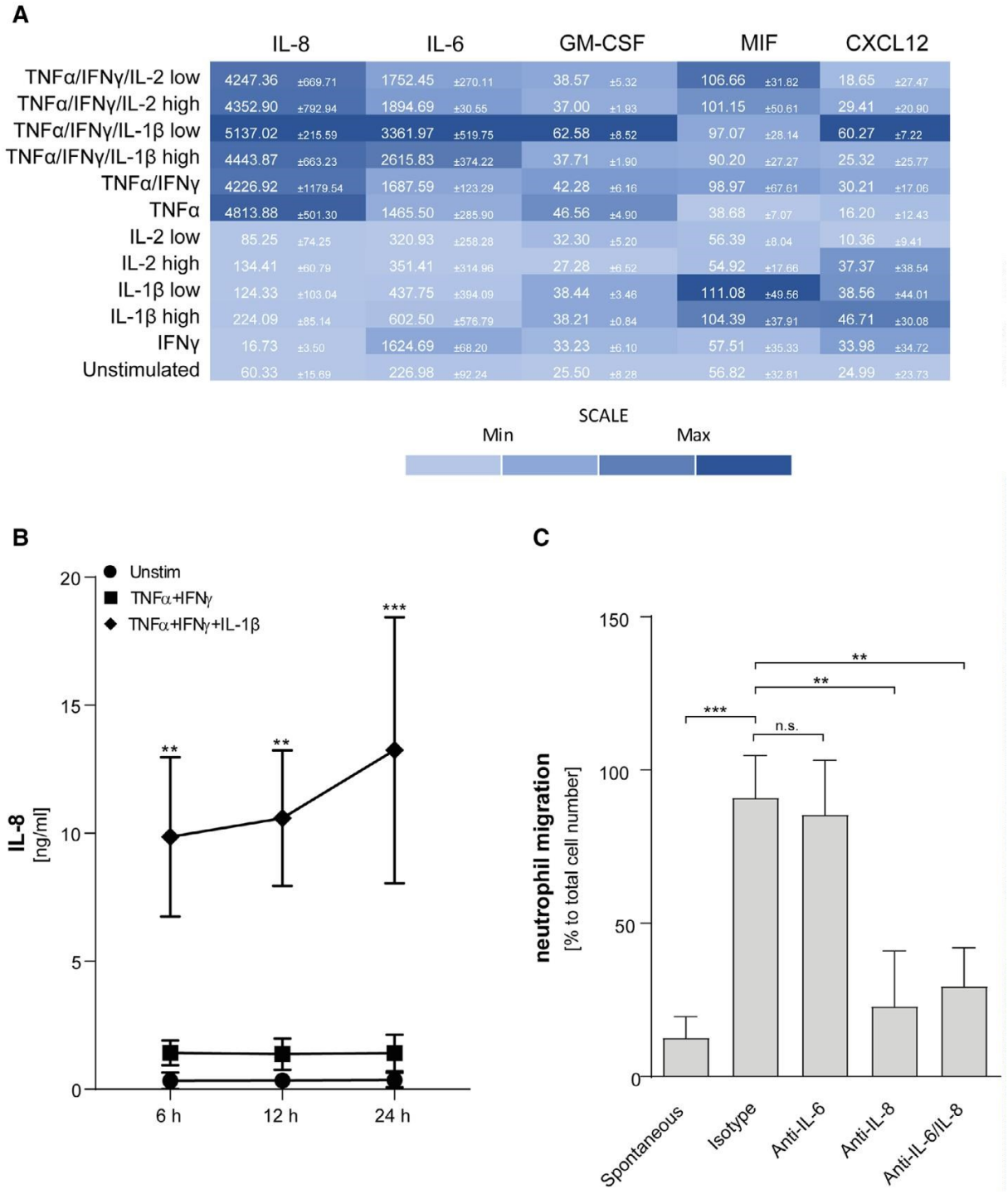
FIGURE 3. Inhibition of NF-kB, p38-MAPK or STAT5 blocks IL-8 release by MSC. MSC from four different donors were independently stimulated with TNFα/IFNγ/IL-1β for 24h, after 30 min pre-incubation with inhibitor NF-kB II or inhibitor NF-kB III in the concentration 3μM, 10μM and 30μM. DMSO was used as solvent control. Stat5-inhibitor and/or p38-MAPK inhibitor SB202190 were added 4h after cytokine stimulation in the concentration 3μM, 10μM and 30μM. SN was collected after 24h and IL-8 was measured by ELISA. TNFα/IFNγ/IL-1β stimulated MSC (plus solvent control) were set as 100%. One-way ANOVA with Tukey's multiple comparison test was used to test statistical significance. Data is shown with the mean as the centre value and error bars indicate SD. P-values as indicated in the figure from left to right: (30μM) *P=.0127, ***P=.0008, ****P<.0001, **** P<.0001; (10μM) **P=.0025, ****P<.0001, *P=.0104; (3μM) *P=.0371, *P=.0104, ***P=.0003.

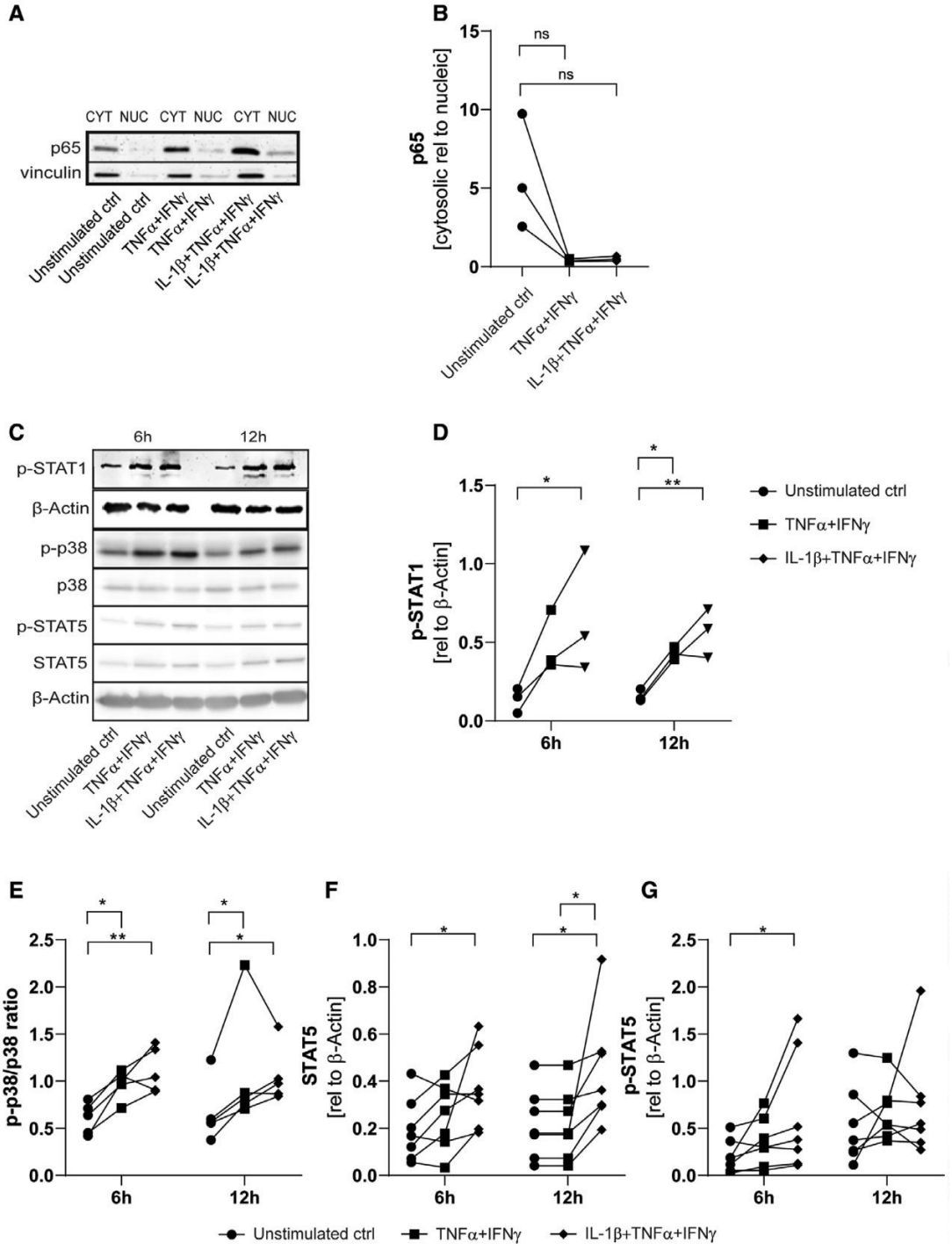
FIGURE 4. Cytokine receptor regulation during multi-cytokine priming. (A) MSC from three different donors were independently stimulated with TNFα/IFNγ or TNFα/IFNγ/IL-1β, cells were harvested after 12h and 24h, and IFN-gamma receptor (IFNR), IL-1 receptor 1 (IL-1R) and TNF receptor Type 1 (TNFR) expression was measured by flow cytometry (delta median = marker expression minus isotype control). Ratio paired parametric t-test was used to test statistical significance. P values from left to right: IFNR, **P=.0012, *P=.0199; IL-1R, **P=.0025, *P=.0177; TNFR, **P=.0052, n.s. P=.076. (B) MSC from four donors were independently pre-stimulated by cytokine combinations for 4h and subsequently stimulated by single cytokines as indicated in the legend and then incubated for additional 18h. SN was collected and IL-8 was measured for each donor by ELISA. Triple cytokine stimulation served as reference and control. One-way ANOVA with Tukey's multiple comparison test was used to test statistical significance. P-Values as indicated from left to right: ***P= .0001, **P=.0021, *P=.0335, ****P<.0001. (C) MSC from three different donors were independently stimulated by TNFα/IFNγ or TNFα/IL-1β and additional IFNγ after 4h, cells were harvested after 24h and IFN-gamma receptor (IFNR) expression was measured by flow cytometry

(delta median = marker expression minus isotype control). Parametric paired t-test was used to test statistical significance of unstimulated versus stimulated conditions. P-Values as indicated from left to right: *P= .0149, ***P=.0002. All data is shown with mean as centre value and error bars indicate SD.

FIGURE 5. MSC primed by IL-1β and TNFα upregulate IFNγR, followed by augmented IFN-γ response. (A) MSC from three different donors were independently stimulated by TNFα/IL-1β in the presence of STAT5-inhibitor (10 μM), p38-MAPK inhibitor SB202190 (10 μM) or inhibitor NF-kB III (10 μM). Cells were harvested after 24h and IFN-gamma receptor (IFNR) expression was measured by flow cytometry (normalised to unstimulated control). Ratio paired parametric t-test was used to test statistical significance, P values from left to right: n.s. P=.1582, n.s. P=.6761, *P= .0146. (B) MSC from three different donors were independently stimulated by TNFα/IL-1β and additional IFNγ was added after 4h. Stat5-inhibitor (10 μM) and/or p38-MAPK inhibitor SB202190 (10 μM) were added together with IFN-γ. SN was collected after 24h and IL-8 was measured for each donor in a single ELISA. Ratio paired parametric t-test was used to test statistical significance. P values from left to right: ***P=.0004, n.s. P=.0671, **P=.0022. (C) MSC from three different donors were independently stimulated by TNFα/IL-1β and additional IFNγ after 4h. Stat5-inhibitor (10 μM) and p38-MAPK inhibitor SB202190 (10 μM) were added together with IFN-γ. SN was collected after 24h total incubation time, the SN from different donors was pooled and 3h neutrophil migration assay was performed. Ratio paired parametric t-test was used to test statistical significance between inhibitor and non-inhibitor groups, P values from left to right: *P=.0387, *P=.0432, *P=.0158. DMSO (0.1%) was used as solvent control in all experiments. All data are shown as mean (centre value) and the error bars represent the SD. (D) PMN were incubated in two-fold diluted conditioned MSC medium for 24h, stained with PE-conjugated annexin-V and 7-AAD and analysed by flow cytometry. All data are shown as mean of four independent experiments. Ratio paired parametric t-test was used to test statistical significance. P values from left to right: ***P=.0006, n.s.=.054, **P=.0019, ***P=.0001, **P=.0031. (E) PMN were pre-stimulated in conditioned MSC medium for 1 h. PMNs were incubated in the presence or absence of Escherichia coli with MOI 2.5, 5, 10, and 20, followed by addition of dihydrorhodamine123. Superoxide anion (O_2^-) release was analysed by flow cytometry. Each panel of the figure shows the mean (centre value) and the errors bars (SD) of 5 independent experiments. (F) PMN were stimulated as in D. Neutrophil SN was collected, and the levels of CCL4 were determined by ELISA. Data are means of four independent

experiments. Ratio paired parametric t-test was used to test statistical significance. P values from left to right: n.s. P=.054, *P=.0197, *P=.0197, *P=.0731. No CCL4 could be detected in the MSC SN that was used for PMN stimulation.


A
IL-8
IL-6
GM-CSF
MIF
CXCL12
TNFα/IFNγ/IL-2 low 4247.36 ±669.71 1752.45 ±270.11 38.57 ±5.32 106.66 ±31.82 18.65 ±27.47
TNFα/IFNγ/IL-2 high 4352.90 ±792.94 1894.69 ±30.55 37.00 ±1.93 101.15 ±50.61 29.41 ±20.90
TNFα/IFNγ/IL-1β low 5137.02 ±215.59 3361.97 ±519.75 62.58 ±8.52 97.07 ±28.14 60.27 ±7.22
TNFα/IFNγ/IL-1β high 4443.87 ±663.23 2615.83 ±374.22 37.71 ±1.90 90.20 ±27.27 25.32 ±25.77
TNFα/IFNγ 4226.92 ±1179.54 1687.59 ±123.29 42.28 ±6.16 98.97 ±67.61 30.21 ±17.06
TNFα 4813.88 ±501.30 1465.50 ±285.90 46.56 ±4.90 38.68 ±7.07 16.20 ±12.43
IL-2 low 85.25 ±74.25 320.93 ±258.28 32.30 ±5.20 56.39 ±8.04 10.36 ±9.41
IL-2 high 134.41 ±60.79 351.41 ±314.96 27.28 ±6.52 54.92 ±17.66 37.37 ±38.54
IL-1β low 124.33 ±103.04 437.75 ±394.09 38.44 ±3.46 111.08 ±49.56 38.56 ±44.01
IL-1β high 224.09 ±85.14 602.50 ±576.79 38.21 ±0.84 104.39 ±37.91 46.71 ±30.08
IFNγ 16.73 ±3.50 1624.69 ±68.20 33.23 ±6.10 57.51 ±35.33 33.98 ±34.72
Unstimulated 60.33 ±15.69 226.98 ±92.24 25.50 ±8.28 56.82 ±32.81 24.99 ±23.73
SCALE
Min
Max
B
Unstim
TNFα+IFNγ
TNFα+IFNγ+IL-1β
IL-8 [ng/ml]
20
15
10
5
0
**
**
***
6 h
12 h
24 h
C
neutrophil migration [% to total cell number]
150
100
50
0
***
n.s.
**
**
Spontaneous
Isotype
Anti-IL-6
Anti-IL-8
Anti-IL-6/IL-8


A
CYT NUC CYT NUC CYT NUC
p65
vinculin
Unstimulated ctrl
Unstimulated ctrl
TNFα+IFNγ
TNFα+IFNγ
IL-1β+TNFα+IFNγ
IL-1β+TNFα+IFNγ
B
p65
[cytosolic rel to nucleic]
ns
ns
Unstimulated ctrl
TNFα+IFNγ
IL-1β+TNFα+IFNγ
C
6h
12h
p-STAT1
β-Actin
p-p38
p38
p-STAT5
STAT5
β-Actin
D
p-STAT1
[rel to β-Actin]
Unstimulated ctrl
TNFα+IFNγ
IL-1β+TNFα+IFNγ
6h
12h
E
p-p38/p38 ratio
F
STAT5
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G
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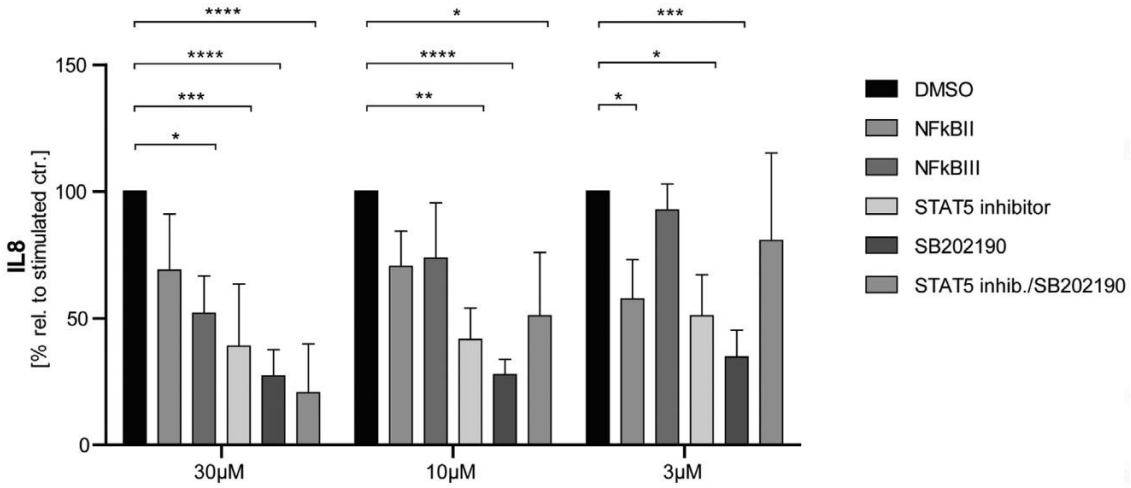
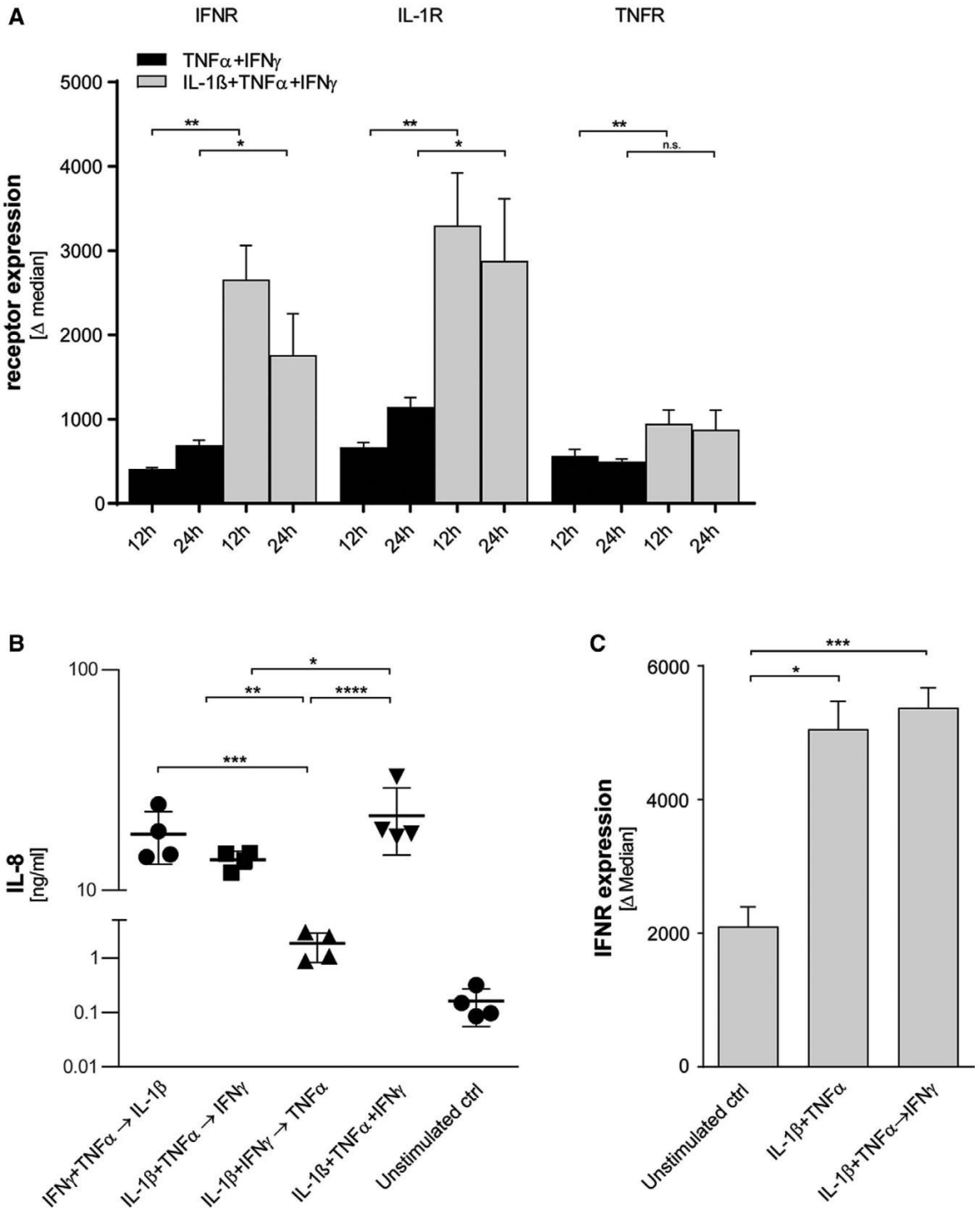
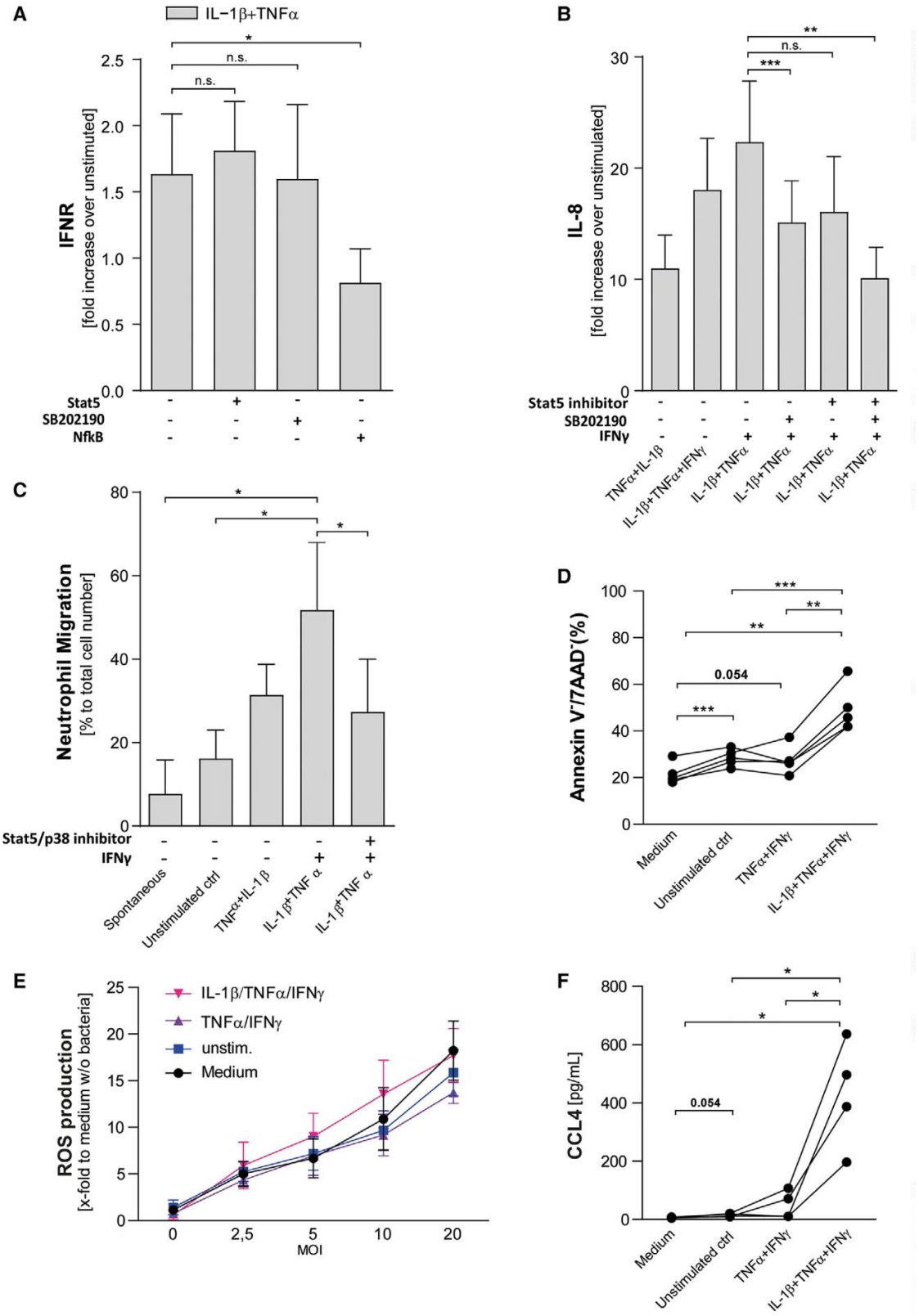
Unstimulated ctrl
TNFα+IFNγ
IL-1β+TNFα+IFNγ


IL8
[% rel. to stimulated ctr.]
150
100
50
0
****
****
***
*
*
****
**
***
*
*
30µM
10µM
3µM
DMSO
NFkBII
NFkBIII
STAT5 inhibitor
SB202190
STAT5 inhib./SB202190


A
IFNR
IL-1R
TNFR
TNFα+IFNγ
IL-1ß+TNFα+IFNγ
receptor expression
[Δ median]
5000
4000
3000
2000
1000
0
**
*
n.s.
12h
24h
B
IL-8
[ng/ml]
100
10
1
0.1
0.01
***
**
****
IFNγ+TNFα → IL-1β
IL-1β+TNFα → IFNγ
IL-1β+IFNγ → TNFα
IL-1ß+TNFα+IFNγ
Unstimulated ctrl
C
IFNR expression
[Δ Median]
6000
4000
2000
0
Unstimulated ctrl
IL-1β+TNFα
IL-1β+TNFα→IFNγ


A
IL−1β+TNFα
IFNR
[fold increase over unstimuted]
n.s.
n.s.
*
Stat5
SB202190
NfkB
B
IL-8
[fold increase over unstimulated]
**
n.s.
***
Stat5 inhibitor
SB202190
IFNγ
TNFα+IL-1β
IL-1β+TNFα+IFNγ
IL-1β+TNFα
C
Neutrophil Migration
[% to total cell number]
Stat5/p38 inhibitor
IFNγ
Spontaneous
Unstimulated ctrl
TNFα+IL-1β
IL-1β+TNF α
D
Annexin V-/7AAD-(%)
0.054
Medium
Unstimulated ctrl
TNFα+IFNγ
IL-1β+TNFα+IFNγ
E
ROS production
[x-fold to medium w/o bacteria]
IL-1β/TNFα/IFNγ
TNFα/IFNγ
unstim.
Medium
MOI
F
CCL4 [pg/mL]
0.054

5.2 Immunological Priming of Mesenchymal Stromal / Stem Cells (MSCs) and their Extracellular Vesicles Augments their Therapeutic Benefits in Experimental Graft-versus-Host Disease via Engagement of PD-1 Ligands

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Keywords: Mesenchymal stromal / stem cells (MSCs), Graft-versus-Host Disease (GvHD), Immunomodulation, Extracellular Vesicles (EVs), Regulatory T cells (Tregs), Cytokine Priming, Programmed Death Ligand System (PD-1 and PD-L1),

None of the authors has any conflict of interest regarding this manuscript

Please note that this version may vary from the final published version due to editorial changes

Abstract

Mesenchymal stromal cells (MSCs) and their extracellular vesicles (EVs) exert profound anti-inflammatory and regenerative effects in inflammation and tissue damage, which makes them an attractive tool for cellular therapies. In this study we have assessed the inducible immunoregulatory properties of MSCs and their EVs upon stimulation with different combinations of cytokines. First, we found that MSCs primed with IFN-γ, TNF-α and IL1β, upregulate the expression of PD-1 ligands, as crucial mediators of their immunomodulatory activity. Further, primed MSCs and MSC-EVs, compared to unstimulated MSCs and MSC-EVs, had increased immunosuppressive effects on activated T cells and mediated an enhanced induction of regulatory T cells, in a PD-1 dependent manner. Importantly, EVs derived from primed MSCs reduced the clinical score and prolonged the survival of mice in a model of graft-versus-host disease. These effects could be reversed *in vitro* and *in vivo*, by adding neutralizing antibodies directed against PD-L1 and PD-L2 to both MSCs and their EVs.

In conclusion, our data reveal a priming strategy that potentiates the immunoregulatory function of MSCs and their EVs. This concept also provides new opportunities to improve the clinical applicability and efficiency of cellular or EV-based therapeutic MSC products.

Introduction

Mesenchymal stromal / stem cells (MSC) are non-hematopoietic, fibroblast-like progenitor cells, capable of differentiating into different mesenchymal tissue lineages, such as chondrocytes, osteoblasts and adipocytes (Pittenger et al., 2019;Viswanathan et al., 2019; Moll et al., 2020;Capilla-González et al., 2022). They are defined by their plastic adherence, the expression of a set of characteristic cell surface markers, but absence of endothelial and hematopoietic cell surface antigens, and their multilineage differentiation capacity (Pittenger et al., 1999;Horwitz et al., 2005b;Dominici et al., 2006b;Pittenger et al., 2019). Originally identified in the bone marrow, they have now been isolated from many vascularized tissue sources and body fluids (Jakob et al., 2010b;Gregoire-Gauthier et al., 2012; Kim et al., 2017;Moll et al., 2019;Cottle et al., 2022;Moll et al., 2022). Considering their broad immunoregulatory capacity towards the innate and adaptive immune system, MSCs are in the focus as a novel therapeutic approach in many inflammation-related diseases (Sherman et al., 2017;Liu et al., 2019;Pedrosa et al., 2019;Ringdén et al., 2022).

MSCs display their multifaceted immunomodulatory properties via both, cell-contact-dependent direct mechanisms and contact-independent paracrine mechanisms, including the induction of anti-inflammatory dendritic cells (DCs) and Tregs (Zhang et al., 2004; Luz-Crawford et al., 2013;Franquesa et al., 2015a;Zhang et al., 2017;Lu et al., 2019). While initial studies have indicated that MSCs are capable of migrating to areas of tissue damage, more recent studies suggest that MSCs often do not reach these sites, but rather accumulate in the lung and spleen and are rapidly cleared from the system (Schrepfer et al., 2007; Duijvestein et al., 2011;Zhu et al., 2013;Moll et al., 2019;Cottle et al., 2022;Moll et al., 2022). Thus, immunomodulation exerted by MSCs is strongly associated with paracrine mechanisms,

e.g. extracellular vesicles (EVs) are suggested as potential mediators of their therapeutic effects (Blazquez et al., 2014;Kordelas et al., 2014;Harrell et al., 2019).

EVs contain a multitude of bio- and immuno-active molecules, such as cytokines, nucleic acids, and other proteins, which in part resemble a comparable molecular spectrum to their parental cells of origin (Fierabracci et al., 2015;Yanez-Mo et al., 2015). Regarding their immunoregulatory activity, MSC-derived EVs exert comparable therapeutic effects akin to the MSCs themselves, (Kordelas et al., 2014;Wang et al., 2016;Alvarez et al., 2018). Compared to their parental cells and conventional MSC-based therapy, the use of MSC-EVs represent a more an easy-to-handle sterile therapeutic tool, whose application also minimizes any risks for patients (Kordelas et al., 2014;Börger et al., 2017;Moll et al., 2019;Cottle et al., 2022; Moll et al., 2022).

The receptor programmed cell death 1 (PD-1) system is a crucial component in the regulation and activation of T cells, as demonstrated by the enhanced susceptibility of PD-1 knockout mice to autoimmune diseases (Nishimura et al., 1998;Salama et al., 2003) and its role in GvHD mice models (Tobin et al., 2013;Fujiwara et al., 2014;Davies et al., 2017). The expression of PD-1 ligand 1 (PD-L1) is reported on non-hematopoietic cells, like MSCs, but also on hematopoietic cells, while PD-1 ligand 2 (PD-L2)-expression is typically found on antigen-presenting cells (APCs), but it is also found to be expressed by MSCs (Yamazaki et al., 2002;Chinnadurai et al., 2014a;Davies et al., 2017). Previous reports have suggested the presence of PD-L1 within EVs and as soluble ‘free’ entities, and in addition as part of soluble cell membrane particles (Davies et al., 2017;Goncalves et al., 2017;Chen et al., 2018).

A big challenge in MSC and EV therapy is to overcome the considerable variations in therapeutic efficiency observed between different donor and manufacturing batches (Moll et al., 2011). Variations in culture conditions, differences in donor and tissue origin, but also variations in isolation and culture procedures can alter the epigenetic profile of MSCs, thus

providing a challenge to generate immunoregulatory MSCs / EVs with consistent properties (Costa et al., 2021). MSC biology itself may provide some important cues to generate higher degree of reproducibility. Indeed, exposure to an inflammatory environment is necessary to fully activate MSCs immunoregulatory function to a more robust level of homogeneity (Petri et al., 2017;Liang et al., 2018).

In on our prior studies to assess the immune response of MSCs to multi-cytokine stimulations (Hackel et al., 2021), we have identified an optimal pro-inflammatory cytokine stimulation approach for MSCs, to be employed prior to their therapeutic application, for generating fully activated MSCs and MSC-EVs with an increased and robust immunoregulatory capacity. Building on our previous studies (Petri et al., 2017;Kordelas et al., 2019), we here used tissue-specific MSCs derived from two different sources, nasal mucosa and human bone marrow, to evaluate their immunomodulatory features and the underlying mechanism in an *in vivo* mouse model.

Ultimately, this method may provide a more efficient and robust therapeutic approach to better standardize MSCs / EV-based therapy of inflammation-related diseases.

Methods

Study approval, isolation and culture of MSCs

The use of human samples was approved by the ethics committee of the medical faculty of the University Duisburg-Essen. Nasal mucosa MSCs, further referred to as “MSCs” in this study, were obtained from the inferior nasal concha of healthy individuals (age 30-70 years) at the Department of Othorhinolaryngology, University Hospital Essen (Essen, Germany). The isolation and culture of MSCs, evaluation of differentiation potential were conducted as described before (Jakob et al., 2010b). MSCs were cultured in DMEM/RPMI-1640 high glucose (50%/50% v/v), supplemented with 2mM L-Glutamine, 100 IU/ml penicillin, 100 mg/ml streptomycin (all ThermoFisher Scientific, Karlsruhe, Germany) and 10% (v/v)

heat-inactivated FCS (Merck/Biochrom, Berlin, Germany). All MSCs used in experiments were between passages 3-6.

Bone-marrow MSCs further referred as “bmMSCs” were kindly provided by Bernd Giebel from the Institute of Transfusion Medicine, University Hospital Essen, Germany, registered as “MSC 41.5”. BmMSCs were originally isolated from bone-marrow aspirates of healthy individuals after informed consent as described before (Kordelas et al., 2014) and acquisition was approved by the ethics committee of the medical faculty of the University Duisburg-Essen. Phenotyping of bmMSCs used in the study was conducted in line with ISCT minimal criteria for MSCs (Dominici et al., 2006b), by evaluating cell-surface marker expression with flow cytometry and trilineage differentiation to validate multipotent differentiation capacity of MSCs (Jakob et al., 2010b). Experiments with bmMSCS were conducted within passage 4-6. BmMSCs were cultured in DMEM low glucose (PAN Biotech, Aidenbach, Germany), supplemented with 10% platelet lysate (kindly provided by the Institute of Transfusion Medicine, University Hospital Essen), 100 U/mL penicillin-streptomycin-glutamine and 5 IU/mL Heparin (Ratiopharm, Ulm, Germany).

Multi cytokine-priming of MSCs and bmMSCs

Cytokine-priming of MSCs and bmMSCs was based on a previously established concept (Hackel et al., 2021). In brief, MSCs and bmMSCs were stimulated in culture medium, with IFN-γ (1000 U/ml; from PeproTech, Hamburg, Germany) and TNF-α (1000 U/ml; Miltenyi, Bergisch Gladbach, Germany) in the present or absence of IL-1β (10 ng/ml; Miltenyi) for 24h at 37°C, 5% CO_2. Afterwards cells were washed twice with PBS, and incubated in culture medium for additional 48 h. Subsequently, MSCs were either processed directly for FACS analysis, co-culture experiments or administered in mouse GvHD models.

Flow cytometric analysis

Following antibodies were used for MSC characterization: CD29-PE (clone MAR4), CD45-V500 (clone HI30, both BD Bioscience, Heidelberg, Germany), CD31-APC-eFlour780 (clone WM59), CD73-PerCP-eFlour710 (clone AD2, both ThermoFisher scientific), CD34-FITC (clone 581), CD90-Brilliant Violet 421 (clone 5E10) and CD105-Pe-Cy7 (clone 43A3, all BioLegend, Koblenz, Germany). After stimulation with IFN-γ/TNF-α +/- IL-1β cells were stained with CD54-APC (clone HA58) CD274-PerCP-eFlour710 (PD-L1, clone MIH1, all ThermoFisher scientific) and CD273-PE-Vio770 (PD-L2, clone MIH18, Miltenyi). Cells were analysed using FACSCanto II flow cytometer and BD FACS Diva Software 8.0. (BD Bioscience)

Isolation and size characterization of extracellular vesicles from MSCs and bmMSCs.

For isolation of MSCs and bmMSCs EVs, cells were cultured and stimulated with IFN-γ/TNF-α +/- IL-1β as described above in Nunc^{TM} High Cell Factory^{TM}. Cell culture supernatants were collected and EVs were purified by differential centrifugations and polyethylene glycol (PEG) precipitation as recently described [49]. EVs isolated from culture medium of $4*10^7$ MSCs or bmMSC that had been conditioned for 48 h was defined as 1 EV unit. MSC-EV size and particle concentration were determined by using nanoparticle tracking analysis by ZetaView (Particle Metrix, Meerbusch, Germany) [49, 50]. ZetaView was calibrated with a polystyrene bead standard of 100 nm (ThermoFisher Scientific). Loaded samples were recorded by video at 11 positions, repeated 5 times. Further settings were Sensitivity: 75, shutter: 75, minimum brightness: 20, minimum size: 5, maximum size: 20 and median value: 20.

Transmission electron microscopy of extracellular vesicles

Transmission electron microscopy of extracellular vesicles was executed in the department of Physical Chemistry, Faculty of Chemistry, University Duisburg-Essen, Essen, Germany. The MSC-EV preparations were diluted 1:10 (1 EV-unit/ml in 10 mM HEPES, 0.9% NaCl) and

subjected to a formvar-coated copper grid. The samples were further incubated with a staining solution of 0.75% Uranyl formate, 6 mM NaOH and dried at room temperature. MSC-EV samples were analysed with a ZEISS EM910 at 120 kV.

SDS-PAGE and Western blot analysis

For SDS-PAGE supernatants and corresponding EV preperations were incubated with SDS sample buffer (pH 6.8, 50 mM Tris, 4% glycerine, 0.8% SDS, 0.04% bromphenol blue and with or without 1.6% β-mercaptoethanol) as described before (Ludwig et al., 2018). Samples were further analysed by wet immunoblotting on nitrocellulose membranes (GE Healthcare) and staining withfollowing antibodies: mouse anti-human CD9 (VJ1, kindly provided by Francisco Sanchez-Madrid), mouse anti-human CD81 (JS-81, BD-Bioscience), rabbit anti-human/mouse/rat HSP70/HSPA1A (R&D Systems, Abingdon, United Kingdom), rabbit anti-human Flotillin-1 (Sigma-Aldrich, St. Loius, USA) and rabbit anti-human CD274 (PD-L1, Pro-Sci-Inc., Poway, USA). Goat anti-rabbit IgGand goat anti-mouse IgG (both HRP-conjugated, Cell Signaling Technology, Danvers, MA, USA) were used as secondary antibodies.

$CD3^+$ T cell proliferation assay

$CD3^+$ T Cells of healthy donors were isolated from peripheral blood mononuclear cells after density-gradient centrifugation via positive selection using human $CD3^+$ MicroBeads (Miltenyi) according to the manufacturer's instructions. After isolation T cells were were labelled with 10 mmol/l Cell Proliferation Dye eFluor 450 (CPDye405) according to the manufacturer's instructions (ThermoFisher Scientifc). To assess the effect of MSC cells on $CD3^+$ T cells, cells were co-cultured in MSC culture medium (see above) with a T-cell:MSC ratio of 2:1 ($0.5*10^5$ $CD3^+$: $0.25*10^5$ MSCs) at 37°C, 5% CO^2. To study the influence of EV from stimulated MSC on T cell proliferation, $0{,}5*10^5$ $CD3^+$ T cells were cultured in the present or absence of 30 µL isolated EV preparations. T-cell proliferation was induced by adding tetrameric antibody-complex ImmunoCult™ Human CD2/CD3/CD28

(StemCell Technologies, Grenoble, France). CPDye405 intensity was analysed by flow cytometry after 4 days of proliferation. Proliferation index calculation is based on dye dilution and was calculated with ModFit LT 3.3 (Verity Software House) according to an algorithm provided by the software. The index of the non-proliferated fraction was subtracted, and the index of T cells without MSC was set as 100%. The proliferation index is the sum of the cells in all generations divided by the computed number of original parent cells theoretically present at the start of the experiment. The proliferation index thus reflects the increase in cell number in the culture over the course of the experiment.

$CD3^+$ Tregs induction assay

After isolation by CD3 microbeads (see above), $CD3^+$ T cell were stimulated with MSC or bmMSC preparations, in the respective culture medium (see above). Treg assay was performed in a 96-well round-bottom plate coated with antibodies against CD3 (10 mg/ml, clone OKT-3; ThermoFisher scientific) and CD28 (2 mg/ml, clone 28.2; Beckman Coulter) for T cell-activating. To assess the effect of MSC cells on $CD3^+$ T cells, cells were co-cultured in a T-cell:MSC ratio of 2:1 ($0.5*10^5$ $CD3^+$: $0.25*10^5$ MSCs) at 37°C, 5% CO2. To test effects of EVs isolated from stimulated MSC $0,5*10^5$ $CD3^+$ T cells were cultured in the present or absence of 30 µl EV preparations. After 3d of culture $CD3^+$ T cells were stained with CD4-APC-Cy7 (clone RPA-T4) CD25-APC(clone NM-A251, both BD-Bioscience), CD127-PE-Cy7 (eBioRDR5, ThermoFisher scientific), and intracellular with FoxP3-FITC (ECH101, both ThermoFisher scientific). Tregs induction were determined with marker expression of $CD4^+$ $CD127^{dim}$ $CD25^+$ $FOXP3^+$ of total $CD4^+$. Cells were analysed with BD FACSCanto II using BD FACS DIVA 8.01 software (BD Biosciences).

GvHD mouse model

Female BalbALB/c and C57BL/6 mice (12-14 weeks old) were purchased from Charles River Laboratory or Janvier Laboratory were housed in a pathogen-free facility of the University Hospital Essen and treated with water containing antibiotics

(0,11 g/l Neomycin, Ampicillin, Vancomycin and Metronidazole). All animal procedures were performed in accordance with the international guidelines for good laboratory practice and the institutional guidelines of the University Hospital Essen approved by the animal welfare committees of North Rhine Westphalia. MHC-mismatched murine HSCT model of GvHD was generated by transplanting CD90.2 depleted bone marrow cells (bm cells) from female C57BL/6 donor-mice into female Balb/c recipient-mice, previously total body irradiated with a dose of 8 Gy (Anderson et al., 2003;Riesner et al., 2016). The recipient female BALB/c mice were reconstituted with $5*10^6$ bm cells from C57BL/6 mice and $0.5*10^6$ naïve $CD4^+$ spleen cells were used to induce GvHD pathology. For CD90.2 depletion of total bone marrow cells after isolation from femur and tibia of C57BL/6 mice, negative selection mouse CD90.2 cell isolation Kit (Miltenyi, Bergisch Gladbach, Germany) according to the manufacturer's instructions was used. Naïve $CD4^+$ spleen cells were isolated from total spleen after erythrocyte depletion with ammonium-chloride-potassium buffer and subsequent negative selection using mouse naïve $CD4^+$ T cell isolation Kit (Miltenyi) according to the manufacturer's instructions. The clinical symptoms of GvHD were assessed with a clinical scoring system (Supplementary Table S1). In long-term experiments, mice were kept until day **58 after** HSCT to analyse long-term clinical follow up and to record survival curves. In short-term experiment, mice were sacrificed on day **11 after HSCT** and the frequency of Tregs in the circulation was determined. Treatment was performed by intravenous injection of 0.03 EV units per mice for three consecutive days or by a single treatment with $5*10^6$ MSC per mice, starting as soon mice regained weight at day 7 or 8 after BMT. Mice were sacrificed when the respective criteria as set out in the institutional and governmental animal welfare guidelines were reached (supplement table). For neutralizing PD-1 ligands in EV preparations, MSC-EVs were pre-incubated for 30 minutes with inhibitory antibodies against CD274 (PD-L1, clone 29E.2A3, BioLegend) (2µg/ml) and CD273 (PD-L2, MIH18, BioLegend) (2µg/ml). To determine non-specific effects of inhibitory antibodies, isotype controls were used at the same

concentrations as the specific antibodies. Unbound antibodies were removed by 100-kDa molecular weight cut-off (Vivaspin®, Sartorius, Göttingen, Germany) centrifugal polyether sulfone membrane ultrafiltration before intravenous injection of EV preparations. Mice were sacrificed when the respective criteria as set out in the institutional and governmental animal welfare guidelines were reached. Animals that died from radiation disease or due to failed engraftment of bone marrow of C57BL/6 donor mice were excluded from experiments. Mice that had to be sacrificed during the experiment due to clinical scoring were continuously recorded with a score of 10.

Swiss role colon analysis

In order to analyse the colon histology of groups within the short-term GvHD model, we employed a previously published technique referred as "Swiss Role" (Moolenbeek and Ruitenberg, 1981). In brief, directly after sacrifice of mice faces was removed by flushing with PBS. The colon was rolled up on a wooden stick to be subsequently fixed in 4% formalin. The fixed preparations were embedded in paraffin for subsequent cutting in 5 μm sections by microtome. Sections were stained with hematoxylin and eosin (HE) and analysed by light microscopy.

Analysis of blood samples

Blood samples were taken from donor C57BL/6 mice at day 1 and from recipient Balb/c mice on day 11 after irradiation. Mice were anaesthetized with isoflurane; blood was drawn from retro-orbital venous and collected in EDTA-tubes and subjected to flow cytometry analysis. Notably, it was not feasible to collect a sufficient quantity of blood samples from every mouse for further analysis, caused by the severe pathology of the GvHD mouse model.

Statistical analysis

All data are shown as means as centre value and errors bars (+/-) SD or SEM as indicated. Data were analysed by paired parametric t-test or by one-way analysis of variance (ANOVA) with Tukey's multiple comparison test. Kaplan–Meier curves were analysed with

Gehan-Breslow-Wilcoxon to compare survival between treatment groups. Data are presented p-values of $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) or $p < 0.0001$ (****) were considered statistically significant.

Results

Enhanced induction of Tregs by primed MSC and their EVs is mediated by PD-1 Ligands *in vitro*.

The MSCs used in the study were characterised in a standard procedure and daily routine in our lab according to ISCT criteria (Dominici et al., 2006b) **(Figure 1 A+B)**. Crucial for our study is the immunomodulatory priming of MSCs, which was previously shown to mediate MSCs immunoregulatory activity and specific cell surface markers (Barrachina et al., 2016;Petri et al., 2017;Liang et al., 2018).

In our previous work we observed that during triple cytokine priming by TNF-α, IL-1β and IFN-γ, the cytokine IL-1β further augmented the well-established immunoregulatory activity of MSCs induced by TNF-α/IFN-γ (Hackel et al., 2021). Based on previous studies, we decided to test for PD-l ligand expression of cytokine primed MSCs, as these surface proteins have been described to be crucial for MSCs' immunoregulatory activity towards T cells (Kronsteiner et al., 2011;Davies et al., 2017). We analysed MSCs of different donors with respect to their responsiveness to strong triple-cytokine priming (With 1000 U/ml of TNFα/IFNγ and 10ng/ml IL-1β). This response we compared to the well-established dual stimulation with TNFα and IFNγ (each 1000 U/ml) (Li et al., 2015).

Interestingly, we found two response patterns of MSCs. First, MSCs compiled in **Figure 1 C-E** (representative FACS histograms shown in **Figure S1 A**) demonstrated a substantial increase in protein expression after triple-cytokine priming compared to the dual priming with TNFα/IFNγ alone. These MSCs were considered "full-responders". In turn,

MSCs, which were not additionally activated by triple cytokine priming (TNFα/IFNγ and IL-1β) and/or showed lower expression of marker proteins in general, were considered as incompletely responsive (**Figure 1 F-H**). Further *in vitro* and *in vivo* experiments were conducted with full responder MSC preparations.

For validation of MSCs immunoregulatory capacity, we tested the same MSC preparations shown in **Figure 1 C-E** and in addition their derived EVs in different immune-functional *in vitro* read-outs based on $CD3^+$ T lymphocytes (**Figure 2 A -F**). Addition of triple-primed MSC or EVs showed the strongest potential to reduce T cell proliferation **(Fig 2 C, D)** and to induce Tregs **(Fig 2E, F)** in CD3 T cell culture assays.

Multi-cytokine priming increased PD-L1 and PD-L2 expression in full responders (**Figure 1 A-C**). As these proteins have shown strong immunoregulatory activity towards T lymphocytes (Nishimura et al., 1998;Salama et al., 2003), as proof of concept, we inhibited the function of PD-L1 and PD-L2 on MSCs and EVs by neutralizing antibodies and analysed effects on T lymphocyte proliferation and induction of Tregs. Interestingly, we could show that PDL blockade restored $CD3^+$ T lymphocyte proliferation in co-culture systems with MSC (**Figure 3 A, B**). Triple-cytokine stimulated MSCs (**Figure 3 C**) and their EVs **(Figure 3 D)** strongly augmented induction of Tregs in our *in vitro* system. This induction of Tregs was strongly reduced in the presence of inhibitory antibodies to PD-1 Ligands. Interestingly, the Treg induction by unstimulated and dual TNFα/IFNγ stimulated MSCs and their EVs was hardly abrogated after PD-L inhibition. Thus, our results support the notion that Treg induction is largely dependent on PD-1/PDL1/2 interaction.

EVs from immunologically primed MSCs ameliorate murine experimental GvHD

In order to test the therapeutic potential and applicability of the immunologically optimized triple primed MSC and their EVs, defined by our functional *in vitro* experiments

(Figure 1-3), we utilized a model of experimental murine GvHD. We conducted the following *in vivo* experiments with full responder MSCs after triple-priming compared to unstimulated MSCs and PBS control.

In this model, GvHD is generated by transplanting CD90.2-depleted bone marrow cells (BM cells) from female C57BL/6 donor-mice into female Balb/c recipient-mice, previously irradiated with a total dose of 8 Gy. The recipient female Balb/c mice were reconstituted with BM cells from C57BL/6 mice and with naïve $CD4^+$ spleen cells to induce GvHD pathology (Riesner et al., 2016). Groups were treated with MSC-EVs (one injection per day for three consecutive days) or with a single injection of MSCs. Triple-primed MSCs showed an activated phenotype (*in vitro*) and, when injected as whole cells, had a lethal effect on mice directly after intravenous injection, most likely caused by the embolization of lung vessels by this highly activated MSCs.

Thus, in this first set of experiments we compared unstimulated MSCs versus MSC-EVs, and EVs from un-primed versus primed MSCs for the other part (**Figure 4**). From all treatments tested, EVs derived from triple-primed MSCs showed the most beneficial effect in a time course of up to 58 days observation time. At the end of the observation time, we observed a significantly decreased clinical GvHD score, shown with the significant lower slope, after application of triple-primed MSC-EVs compared to all other treatment groups (**Figure 4).**

Additionally, also the overall survival was substantially increased in the group treated with triple primed MSC-EVs (data not shown). Of note, compared to PBS control, the un-primed / resting MSCs (cells) showed a transient decrease in the clinical score directly after treatment (**Figure 4**; at days 8-20). In our priming set-up, only effects of triple-primed MSC-EVs and not primed MSCs (cells) could be analysed as mentioned before.

Next, we considered that PD-L1 and PD-L2 are involved in down-regulating T cell effector function mediating a beneficial therapeutic effect *in vivo*, based on our *in vitro* experiments (compare **Figure 3**). To test this, we treated one group with triple-primed MSC-EVs that were pre-incubated with antibodies directed against PD-L1 and PD-L2 before injection. The second group received primed MSC-EVs pre-incubated with the corresponding isotypes and the control group was treated with PBS + isotype. The unbound antibodies were then removed by 100-kDa molecular weight cut-off (MWCO) centrifugal polyether sulfone membrane ultrafiltration before intravenous injection.

In accordance with the previous results, treatment with EVs generated from triple- primed MSCs decreased the clinical score long term (**Figure 5 A**). Similar results were obtained when Kaplan-Meier survival analysis was applied (**Figure 5 B**). Importantly, the neutralization of PD-1 ligands by blocking antibodies largely abrogated the therapeutic effect (**Figure 5 A and B**) suggesting that upregulation of PD-1 ligands substantially contributes to the enhanced therapeutic efficacy of EVs generated from triple-primed MSCs.

In published work, many MSC-based cellular therapies rely on MSC isolated from bone-marrow. Therefore, in a subsequent experiment we aimed to translate our findings based on triple-primed nasal mucosa MSC-EVs to MSC-EVs derived from bone marrow. The bmMSC-EV preparation showed a strong Treg induction *in vitro* which could be significantly enhanced by triple-priming of bmMSCs **(Figure 6 A).** Neutralizing antibodies against PD-1 ligands led to significant decrease of Treg induction **(Figure 6 A)**. In a final series we tested bmMSC-EVs in a short-term GVHD model. Such short-term model enabled us to avoid early death of mice, high clinical scores and offered the possibility to obtain tissue material and peripheral blood from all experimental animals for full comparative analysis between experimental groups.

Using this approach, we found that triple-primed bmMSC-EVs show a similar beneficial therapeutic effect to MSC-EVs from nasal mucosa **(Figure 6 B and C),** thus demonstrating that this mechanism is conserved for MSCs isolated from different adult tissue reservoirs. These data demonstrate that immunological priming augments therapeutic efficacy of MSC-EVs from different tissue sources. Interestingly, and despite clear differences of clinical scores in treatment groups, the gut pathology as analysed by swiss role technology, was not affected by MSC therapy (Supplement Figure S3).

In addition, the short-term model enabled us to analyse the $CD4^+$ $FoxP3^+$ T lymphocytes in mice blood after scarification at the end of experiment by flow cytometry. The group treated with triple-primed bmMSC-EVs demonstrated the strongest induction of Tregs followed by the unprimed bmMSC-EVs **(Figure 6 D).** Tregs might be key cells in maintaining the therapeutic effect in primed bmMSC-EV treated group.

Discussion

MSC-EVs often recapitulate the immunoregulatory properties of their "parent" cells (Luz-Crawford et al., 2013;Tobin et al., 2013;Blazquez et al., 2014;Kordelas et al., 2014). However, EVs lack the full ability of their parental cells to respond to external signals and thus can only deliver signals and effector molecules already present in their membrane or lumen when generated from their cell of origin (Yanez-Mo et al., 2015). Due to the rather short survival of MSCs in the host (Schrepfer et al., 2007;Zhu et al., 2013;Moll et al., 2019;Moll et al., 2020;Cottle et al., 2022;Moll et al., 2022), it is also questionable whether MSC always receive sufficient priming signals for full immune activation. Against this background we wanted to develop new priming protocols that robustly enhance the immunoregulatory capacity of MSC-EVs ante partum/prior to therapeutic application. In order, to generate "immune enhanced" MSCs we established a triple-cytokine priming protocol, which enhanced the

expression of immunoregulatory proteins associated with MSC's migration and T lymphocyte suppressor function via the PD-1 pathway.

The heterogeneity of MSC therapeutic efficiency, caused by differences in donor and tissue origin as well as isolation and culture procedures, makes it challenging to produce immunoregulatory MSCs with reproducible properties (Moll et al., 2019; Moll et al., 2020;Capilla-González et al., 2022;Cottle et al., 2022;Moll et al., 2022;Ringdén et al., 2022). Neither searching for surrogate markers to predict MSCs immunoregulatory capacity nor producing immortalized MSCs has led to production of MSC-EVs with a robust and reproducible immunoregulatory properties (Phinney, 2012). Importantly, within this study we demonstrate that in particular triple-cytokine priming and pre-testing of MSC-EV preparations improves their immunoregulatory properties and can partly overcome MSC heterogeneity and that of their EVs. Nevertheless, as stated and demonstrated by Kordelas et. al. the recipient-specific response to primed MSC-EVs has to be elucidated and is of crucial importance (Kordelas et al., 2019). Interestingly, we were able to demonstrate that EVs from triple-primed mucosal tissue and bone marrow MSCs significantly increased Treg induction *in vitro* and showed the strongest therapeutic capacity *in vivo*.

Pro-inflammatory stimulation of MSCs has been previously reported to increase PD-1 ligand expression and results in an enhanced suppression of T cell effector function (Chinnadurai et al., 2014a;Li et al., 2015;Chen et al., 2018;Liang et al., 2018; Wobma et al., 2018;Zhang et al., 2018). It has also been demonstrated that PD-L1 and PD-L2 function in unison to immune regulate T cells and promote Tregs induction (Francisco et al., 2009;Davies et al., 2017). Here, we demonstrate that EVs derived from triple-primed MSCs provide an enhanced clinical outcome in a murine GvHD model, and that this therapeutic effect is at least partly mediated by PD-1 ligands. Our data also support a crucial role of PD-1 ligands on MSCs and MSC-EVs in mediating Treg induction. Of note, TGF-β is abundantly found on

EVs and has also shown to immune regulate T cell effector function by inducing Tregs (Alvarez et al., 2018). Interestingly, in work related to this study, we found a significant upregulation of TGF-β secretion by MSCs primed by the same multi-cytokine combination used within this study (Hackel et al., 2021).

Stimulation of MSCs leads to enhanced expression of adhesion molecules and to changes in cellular morphology (Wen et al., 2014;Barrachina et al., 2016). These changes may pose significant risks and side effects especially during intravenous application in the course of cellular therapy (Forslow et al., 2012). In our study, the application of stimulated MSCs also showed a lethal outcome directly after injection in 4 of 5 mice in our GvHD model (data not shown, **Figure 4**), most likely caused by the embolization of lung vessels by highly activated MSCs, (Moll et al., 2019;Cottle et al., 2022;Moll et al., 2022;Ringdén et al., 2022). Fatal embolism was described for transfused human decidual stromal cells before in a likely GvHD mouse model (Sadeghi et al., 2015). These considerations suggest that MSC-EVs may represent a safer and more feasible therapeutic option to prevent therapy-related death (Forslow et al., 2012;Blazquez et al., 2014;Kordelas et al., 2014;Börger et al., 2017). Both, in MSC- and MSC-EV-therapy for severe steroid-refractory acute GvHD, the risk for pneumonia-related and mould infection-related death is increased. However, it stays unclear whether these infections owing to the immune-suppressive effect of the steroid therapy, to the immune-regulatory effect of MSC/MSC-EV or occurring simply by stochastic risk due to the prolonged survival of patient treated with MSC/MSC-EV *per se* (Forslow et al., 2012;Kordelas et al., 2019;Xu et al., 2022).

Conclusion

In this report we tested dual and triple pro-inflammatory stimulation of MSCs to robustly increase the immunoregulatory properties and in turn reduce the functional heterogeneity of the parental MSCs and their derived EVs and to study the underlying mechanisms of action in a well-established preclinical GvHD *in vivo* model. Importantly,

triple-primed MSCs and their EVs, displayed enhanced therapeutic efficiency, in a PD-1 ligand dependent manner.

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Conflict of interest statement

The authors do not have any conflicts of interest to report.

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Figure Legends

Figure 1: Priming with TNFα, IFNγ and IL-1β significantly increased expression of immuno- modulatory surface proteins of distinct MSCs. **(A+B)** Routine phenotyping after isolation in 2nd passage. **(A)** Flow cytometry analysis of MSCs. Data are shown as an overlay histogram: isotype control (gray) and specific cell-surface markers (white). Cells were labelled with antibodies against CD29, CD31, CD34, CD45, CD73, CD90, and CD105. Dead cells were excluded by live/dead staining. Representative experiments of MSCs in this study. **(B)** Trilineage differentiation of MSCs. (I+II) Adipogenic differentiation after 14 days of cultivation with standard culture medium or adipogenic induction medium, sudan III staining (arrow: fat vacuoles); nuclei staining with hematoxylin. (III+IV) Osteogenic differentiation after 21 days of cultivation with standard culture medium or osteogenic induction medium, alizarin red staining. (V+VI) Chondrogenic differentiation after 21 days of cultivation in cultivation with standard culture medium or chondrogenic induction medium, alician blue staining. Representative results from MSCs used in the study. **(C-H)** MSCs were stimulated by TNFα (1000 U) and IFNγ (1000 U) or by TNFα (1000 U) and IFNγ (1000 U) in combination with IL-1β (10ng/ml). (C+F) PD-L1, **(D+G)** PD-L2, (E+H) ICAM-1 expression were measured by flow cytometry, fluorescence mean intensity (MFI): Marker expression minus isotype. **(C-E) "Full responder"** MSCs show further immunoactivating PD-1 ligand expression after triple-priming **(D1-D3, in blue)** compared to **(F-H), (D4-D6).** Paired t-test were used to test statistical significance ($p < 0.05$ considered as significant), data are means (n=3). Data is shown as individual MSC donors.

Figure 2: T cell effector function is strongly suppressed via direct cell-cell contact and by MSC-EVs after TNFα, IFNγ and IL-1β priming. (A) Schematic overview of $CD3^+$ proliferation assay. MSCs were primed for 24h with TNFα/IFNγ or by TNFα /IFNγ in combination with IL-1β, , cells were washed and cultured in media for additional 48h. Fresh isolated

For Tcell proliferation assay $CD3^+$ repsonder cells were stimulated with T cell-activating tetrameric antibody-complex CD2/CD3/CD28 and incubated for 4d in the present or absence of different conditioned MSC preparations. **(B)** $CD3^+$ proliferation measured via dilution of proliferation dye by flow cytometry and proliferation index as indicated below was calculated via Modfit software. **(C)** Co-culture of $0.5*10^5$ $CD3^+$ and $0.25*10^5$ full responder MSCs ($CD3^+$ n = 3), (D) $0.5*10^5$ $CD3^+$ incubated with EVs isolated from conditioned media of full responder MSCs (MSC-EV n=3; $CD3^+$ n=3). Paired t-test was used to test statistical significance ($P < 0.05$ considered as significant), data is shown as centre value: mean; error bars: SD. **(E-F)** For Treg induction assays, MSCs were primed for 24h withTNFα and IFNγ or TNFα, IFNγ and IL-1β SN, cells were washed and additionally incubated in media for additional 48h. Freshly islolated $CD3^+$ T cells were incubated for 3d with different MSC preperations. T cells were activated with plate bound antibodies CD3 (1mg/mL) and CD28 (2µg/mL). **(E)** Co-culture of $0.5*10^5$ $CD3^+$ T cells and $0.25*10^5$ MSCs selected for subsequent *in vivo* assays, ($CD3^+$ n = 3). **(F)** $0.5*10^5$ $CD3^+$ T cells incubated with EV isolated from conditioned media of full responder MSCs (MSC-EV n = 3; $CD3^+$ n = 3). Frequency of Tregs was detected by flow cytometry with MFI marker expression of $CD4^+$ $CD127^{dim}$ $CD25^+$ $FOXP3^+$ of total $CD4^+$. Paired t-test was used to test statistical significance ($p < 0.05$ considered as significant), Data is shown as centre value: mean; error bars: SD.

Figure 3: Suppression of $CD3^+$ T Cells by primed MSCs/MSC-EVs is mediated by PD-1 Ligands. $CD3^+$ isolated from healthy donors were incubated with MSCs/MSC-EVs primed by

TNFα and IFNγ or by TNFα and IFNγ in combination with IL-1β and with inhibitory antibodies against PD-L1 (2µg/ml) and PD-L2 (2µg/ml) or isotype control (mIgG1/2a). **(A-B)** $CD3^+$ proliferation measured via proliferation dye by flow cytometry. $CD3^+$ T Cells cultured with **(A)** primed MSC or **(B)** MSC-EVs. **(C-D)** Tregs were determined with MFI marker expression of $CD4^+$ $CD127^{dim}$ $CD25^+$ $FOXP3^+$ of total $CD4^+$ by flow cytometry. $CD3^+$ T Cells were cultured with **(C)** MSCs or **(D)** incubated with MSC-EVs. Paired t-test was used to test statistical significance ($p < 0.05$ considered as significant), MSC, n = 3. data is shown as centre value: mean; error bars: SD.

Figure 4: Primed MSC-EVs show long-term beneficial therapeutic effects compared to unprimed MSCs. Balb/c mice were lethally irradiated (day 0) and injected with CD90.2 depleted bone marrow cells and naïve CD4 cells from C57BL/6J mice to induce GvHD (day 1). Treatment with MSC-EV was performed at **day 9, 10 and 11. MSC cells were injected at day 9.** Time flowchart of clinical score. Day of deaths and remaining mice per group as indicated. Numbers at the end of linear regressions indicate the slope. P value indicates statistical differences between the groups, [n.s.] on day 13, indicates no significant difference between the groups two days after last treatment, One-way ANOVA with Tukey's multiple comparison test was used to test statistical significance. Data is shown as centre value: mean error bars: SEM**.**

Figure 5: Inhibition of PD-Ligands on primed MSC-EVs abrogates beneficial therapeutic effects. Balb/c mice were lethally irradiated (day 0) and injected with CD90.2 depleted bone marrow cells and naïve CD4 cells from C57BL/6J mice to induce GvHD (day 1) and treated with MSC-EVs of unstimulated MSCs or TNFα, IFNγ and IL-1β stimulated MSCs at day 8, 9 and 10 with primed MSC-EVs pre-incubated with inhibitory PD-1 ligand antibodies or isotype control. **(A)** Time flowchart of clinical score. Data is shown as centre value: mean; error bars: SEM. P value indicates statistical differences between the groups until day 24

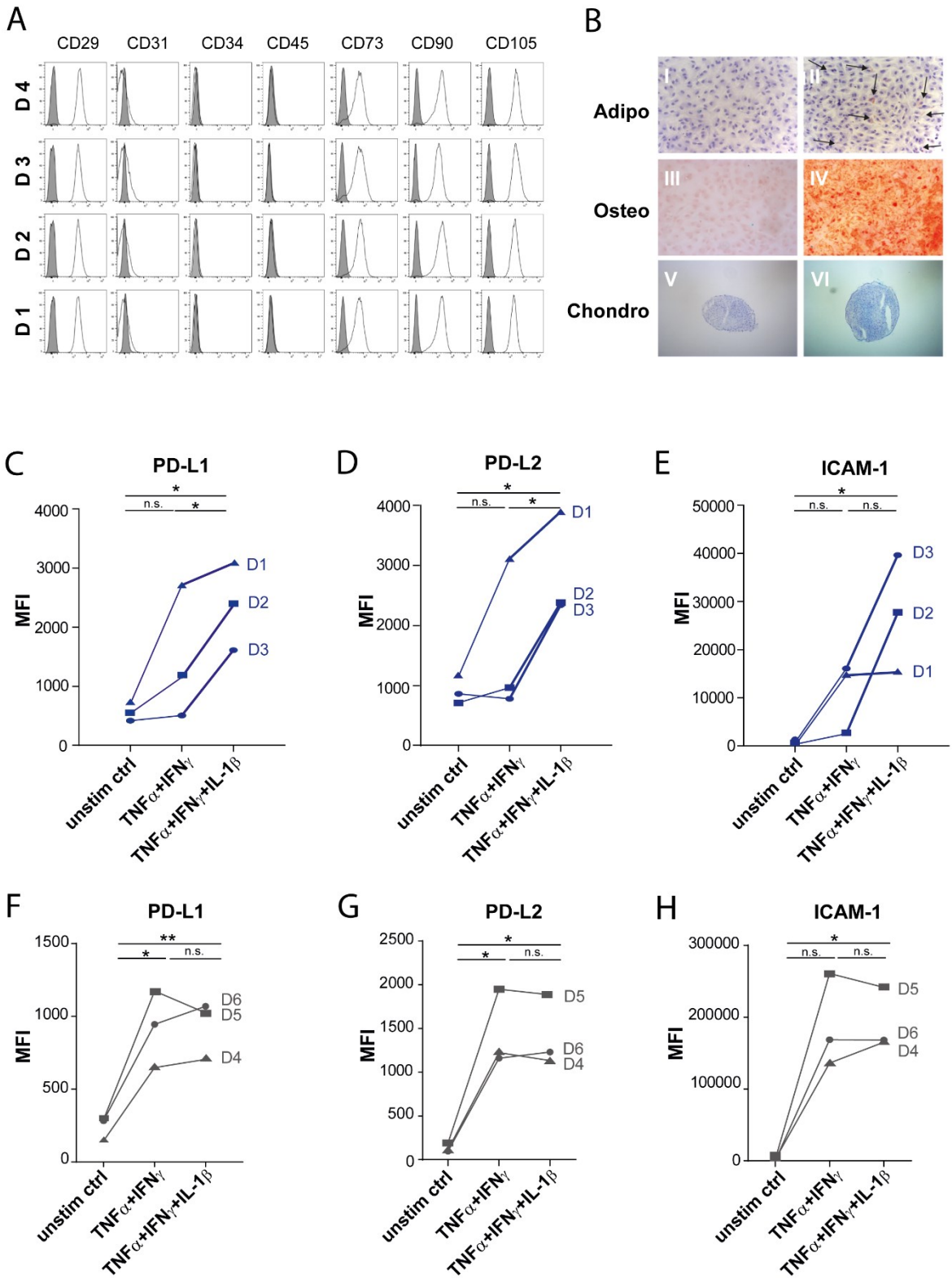
analysed by linear regression. **[*]** Indicates p=0,026 significant difference between triple-primed+isotype

MSC-EV treated and PBS+isotype treated group two days after last treatment on day 12. **(B)** Kaplan Meier survival curve. n = 8; MSC-EV stimulated + anti-PD-L antibody n = 8; MSC-EV stimulated + isotype control, n = 5. P-value indicates statistical difference for survival between treatment groups, Gehan-Breslow-Wilcoxon test was used to test statistical significance.

Figure 6: Therapeutic effects can be confirmed with EVs from bone-marrow MSCs (bmMSCs). (A) Treg induction assay. $CD3^+$ T cells isolated from healthy donors were incubated with EVs of unstimulated bmMSCs or TNFα, IFNγ and IL-1β primed bmMSCs. Assay was performed in the presence of inhibitory antibodies against PD-L1 (2µg/ml) and PD-L2 (2µg/ml) or isotype control (mIgG1/2a). Frequency of Tregs were determined by MFI marker expression of $CD4^+$ CD127dim $CD25^+$ $FOXP3^+$ of total $CD4^+$ by flow cytometry. $CD3^+$ T cells from 4 different donors were tested with 3 different independently cultured and precipitated EV preparations of bmMSC 41.5 batch. Mixed reaction (REML) test was used to test statistical significance, ($) P= n.s., (#) P=0,0248. **(B-D)** Balb/c mice were lethally irradiated (day 0) and injected with CD90.2 depleted bone marrow cells and naïve CD4 cells from C57BL/6J mice to induce GvHD (day 1) and treated with bmMSC-EVs at day 7, 8 and 9. **(B)** Clinical score at day 11 of experiment. One-way ANOVA with Tukey's multiple comparison test was used to test statistical significance. Data is shown as centre value: mean; error bars: SD. **(C)** Time flowchart of clinical score. Data is shown as centre value: mean; error bars: SEM. Values on linear regressions indicate the slope. P value indicates statistical differences between the groups analysed by linear regression. **(B-C)** Combined data from two independent animal experiments; bmMSC-EVs unstimulated, n = 9; bmMSC-EV primed, n = 9; PBS control, n = 6. **(D)** Percentage of $CD4^+$ $FOXP3^+$ Tregs in whole blood after sacrifice.

Mice with insufficient blood for further processing are excluded. One-way ANOVA with Tukey's multiple comparison test was used to test statistical significance. Data is shown as centre value: mean; error bars: SD. bmMSC-EVs unstimulated, n = 7; bmMSC-EVs primed, n = 9; PBS control, n = 5.

Figure 1


A
CD29
CD31
CD34
CD45
CD73
CD90
CD105
D 4
D 3
D 2
D 1
B
Adipo
Osteo
Chondro
I
II
III
IV
V
VI
C
PD-L1
MFI
4000
3000
2000
1000
0
n.s.
*
*
D1
D2
D3
unstim ctrl
TNFα+IFNγ
TNFα+IFNγ+IL-1β
D
PD-L2
MFI
4000
3000
2000
1000
0
n.s.
*
*
D1
D2
D3
unstim ctrl
TNFα+IFNγ
TNFα+IFNγ+IL-1β
E
ICAM-1
MFI
50000
40000
30000
20000
10000
0
n.s.
*
n.s.
D3
D2
D1
unstim ctrl
TNFα+IFNγ
TNFα+IFNγ+IL-1β
F
PD-L1
MFI
1500
1000
500
0
**
*
n.s.
D6
D5
D4
unstim ctrl
TNFα+IFNγ
TNFα+IFNγ+IL-1β
G
PD-L2
MFI
2500
2000
1500
1000
500
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*
n.s.
D5
D6
D4
unstim ctrl
TNFα+IFNγ
TNFα+IFNγ+IL-1β
H
ICAM-1
MFI
300000
200000
100000
0
*
n.s.
n.s.
D5
D6
D4
unstim ctrl
TNFα+IFNγ
TNFα+IFNγ+IL-1β

Figure 2

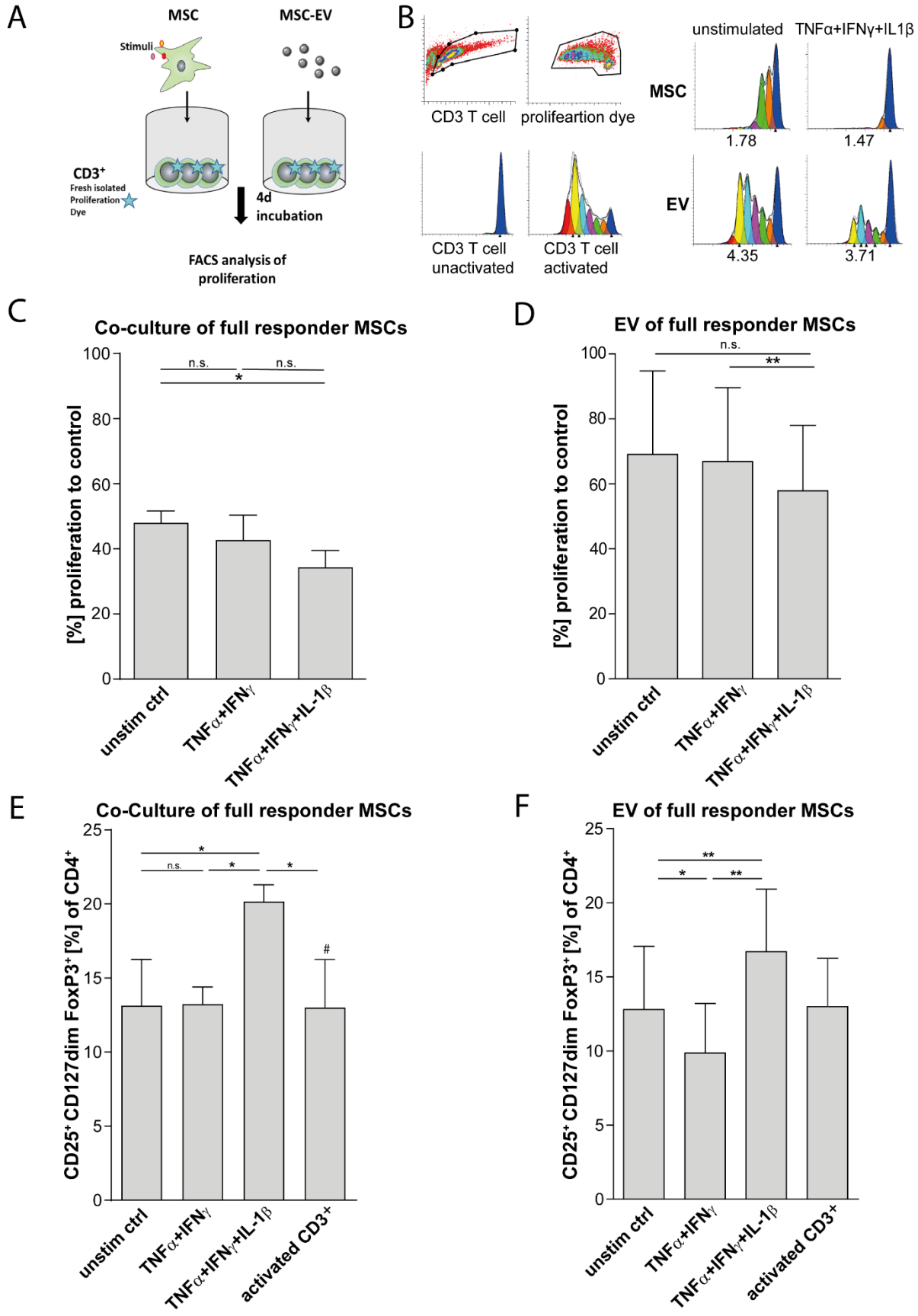

A
MSC
MSC-EV
Stimuli
CD3+
Fresh isolated
Proliferation
Dye
4d
incubation
FACS analysis of
proliferation
B
CD3 T cell
prolifeartion dye
unstimulated
TNFα+IFNγ+IL1β
MSC
1.78
1.47
EV
4.35
3.71
CD3 T cell
unactivated
CD3 T cell
activated
C
Co-culture of full responder MSCs
[%] proliferation to control
n.s.
n.s.
*
unstim ctrl
TNFα+IFNγ
TNFα+IFNγ+IL-1β
D
EV of full responder MSCs
[%] proliferation to control
n.s.
**
unstim ctrl
TNFα+IFNγ
TNFα+IFNγ+IL-1β
E
Co-Culture of full responder MSCs
CD25+CD127dim FoxP3+ [%] of CD4+
n.s.
*
#
unstim ctrl
TNFα+IFNγ
TNFα+IFNγ+IL-1β
activated CD3+
F
EV of full responder MSCs
CD25+CD127dim FoxP3+ [%] of CD4+
*
**
unstim ctrl
TNFα+IFNγ
TNFα+IFNγ+IL-1β
activated CD3+

Figure 3

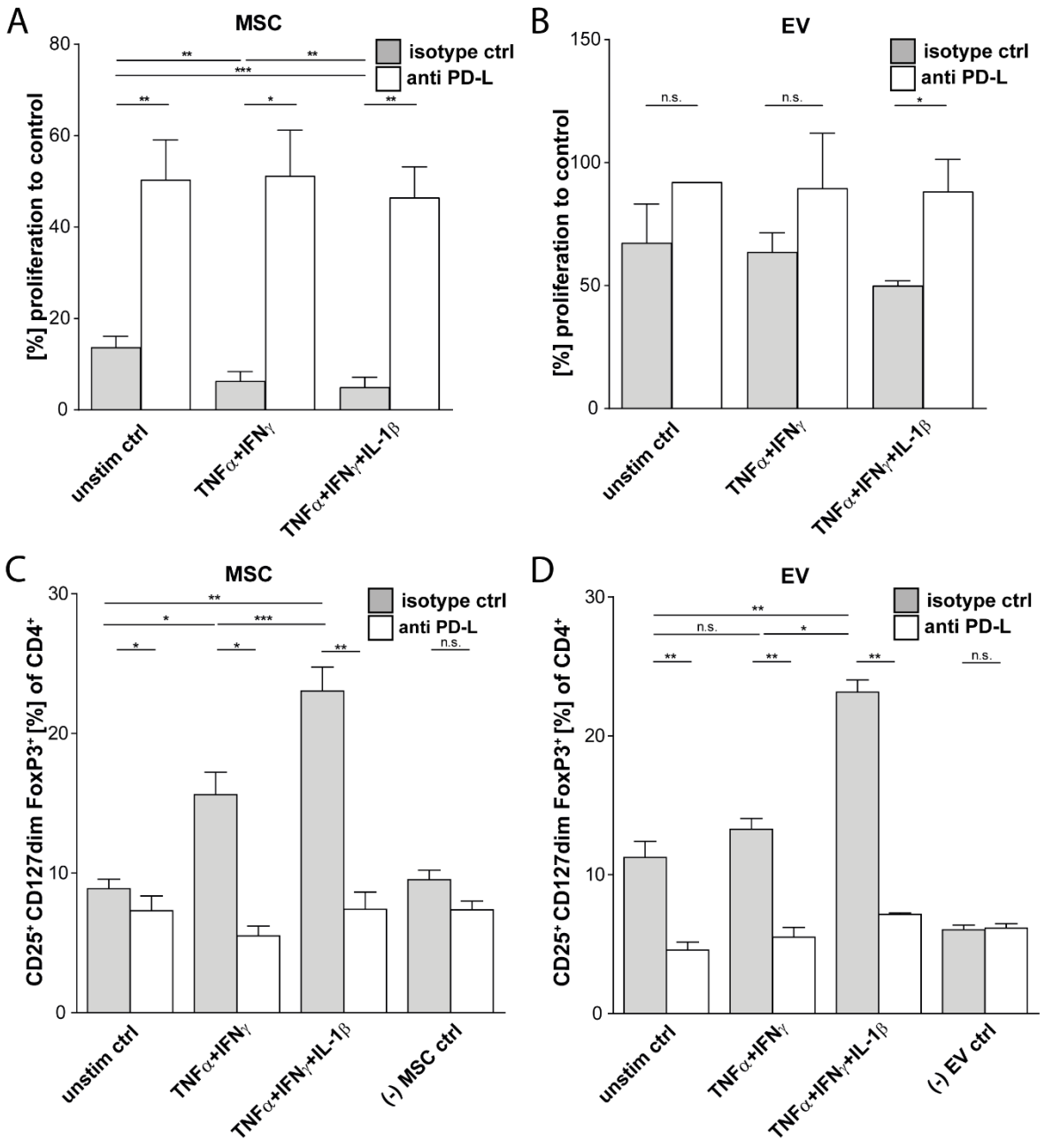

A
MSC
isotype ctrl
anti PD-L
[%] proliferation to control
unstim ctrl
TNFα+IFNγ
TNFα+IFNγ+IL-1β
B
EV
isotype ctrl
anti PD-L
[%] proliferation to control
n.s.
unstim ctrl
TNFα+IFNγ
TNFα+IFNγ+IL-1β
C
MSC
isotype ctrl
anti PD-L
CD25+ CD127dim FoxP3+ [%] of CD4+
n.s.
unstim ctrl
TNFα+IFNγ
TNFα+IFNγ+IL-1β
(-) MSC ctrl
D
EV
isotype ctrl
anti PD-L
CD25+ CD127dim FoxP3+ [%] of CD4+
n.s.
unstim ctrl
TNFα+IFNγ
TNFα+IFNγ+IL-1β
(-) EV ctrl

Figure 4

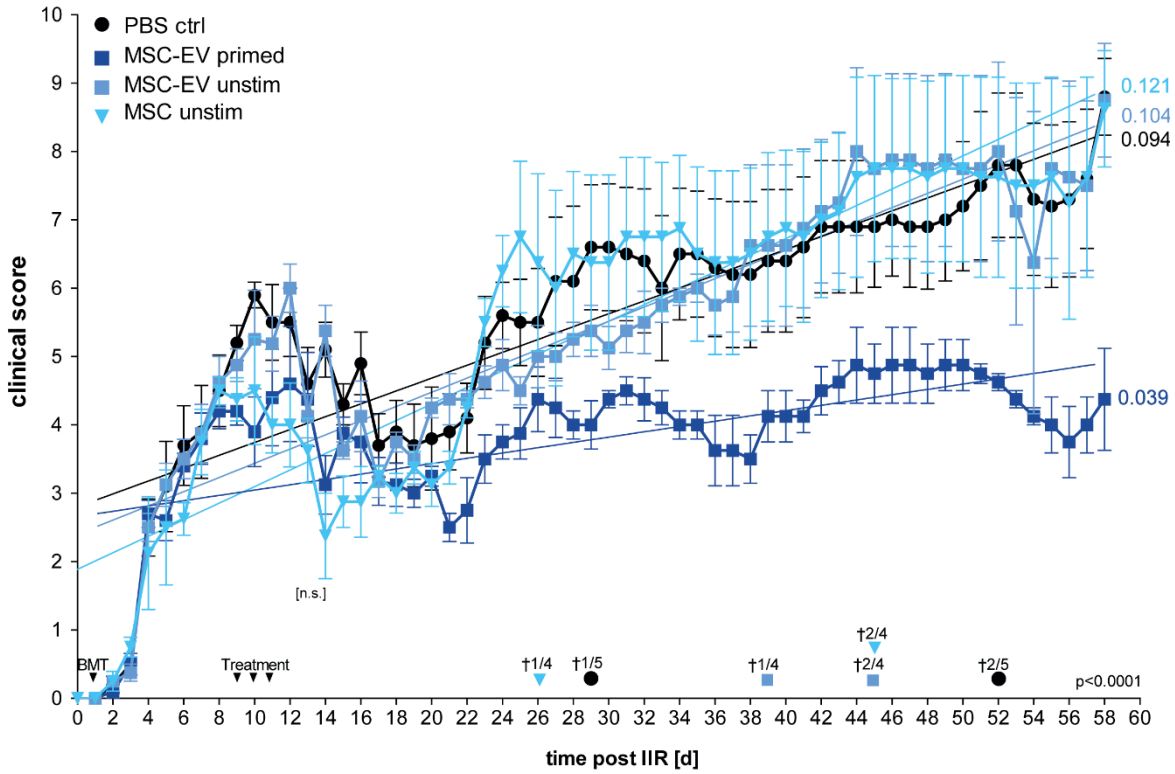

PBS ctrl
MSC-EV primed
MSC-EV unstim
MSC unstim
clinical score
time post IIR [d]
0.121
0.104
0.094
0.039
[n.s.]
BMT
Treatment
†1/4
†1/5
†1/4
†2/4
†2/4
†2/5
p<0.0001

Figure 5

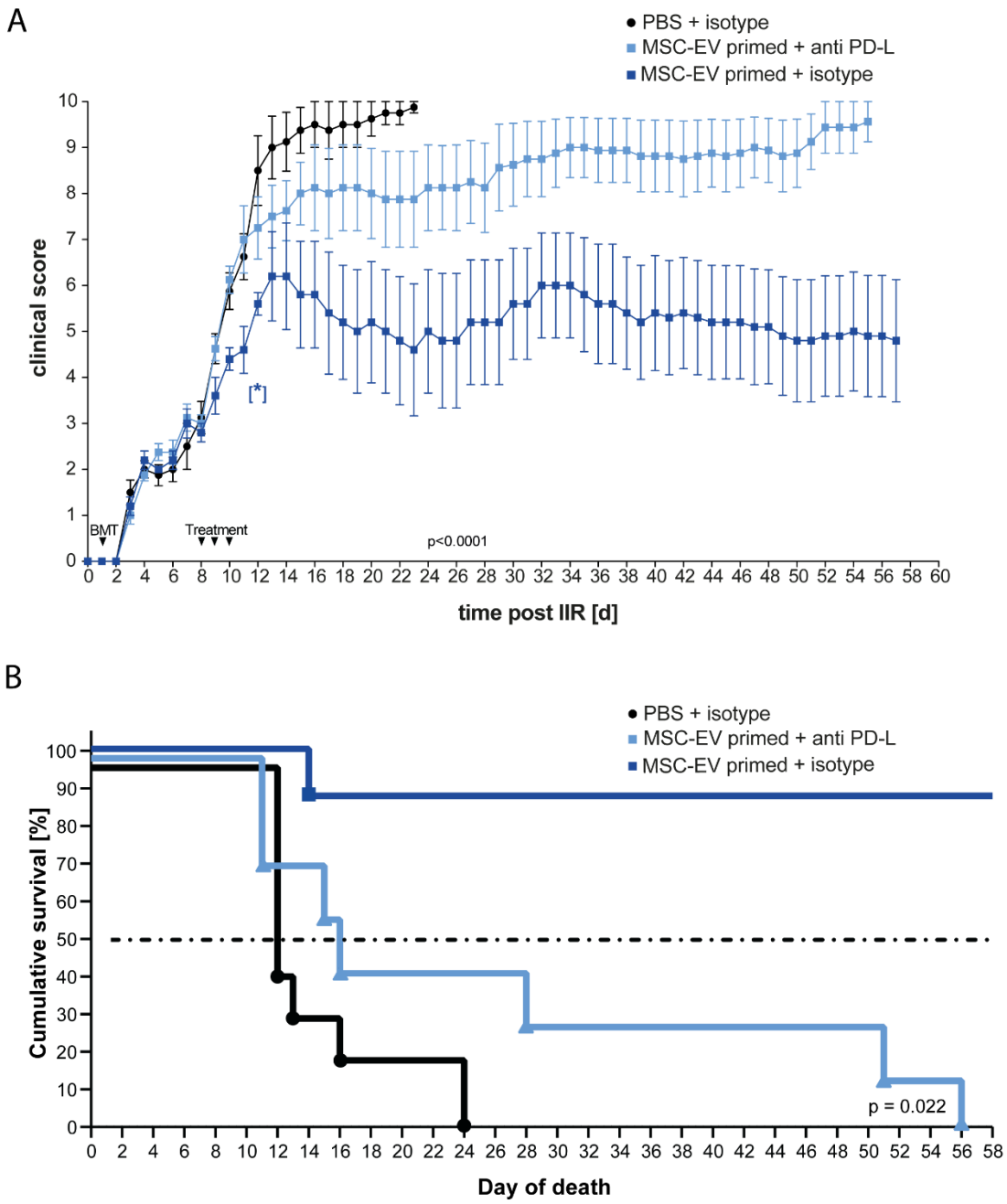
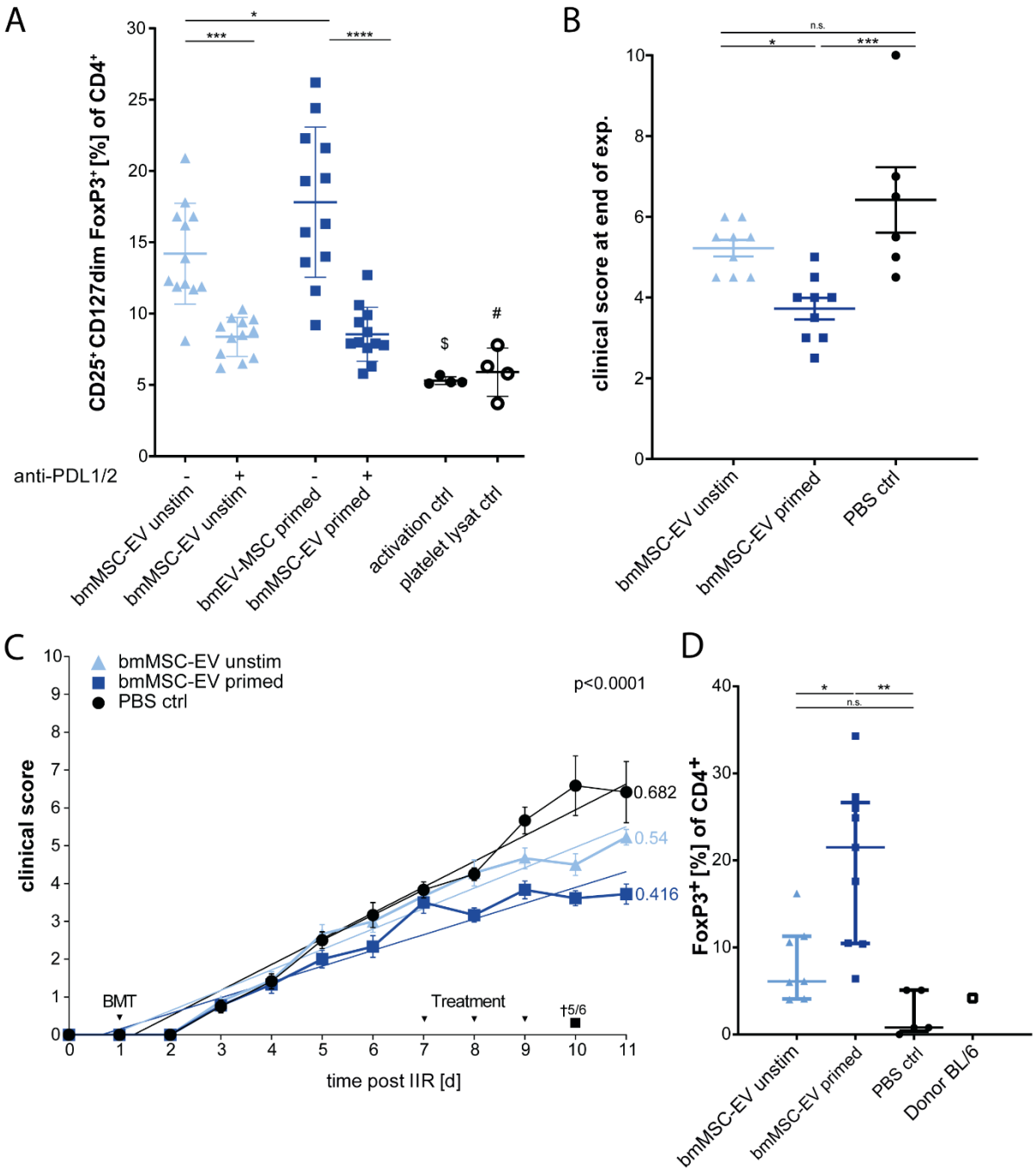

A
PBS + isotype
MSC-EV primed + anti PD-L
MSC-EV primed + isotype
clinical score
time post IIR [d]
BMT
Treatment
[*]
p<0.0001
B
PBS + isotype
MSC-EV primed + anti PD-L
MSC-EV primed + isotype
Cumulative survival [%]
Day of death
p = 0.022

Figure 6


A
CD25+ CD127dim FoxP3+ [%] of CD4+
anti-PDL1/2
bmMSC-EV unstim
bmMSC-EV unstim
bmEV-MSC primed
bmMSC-EV primed
activation ctrl
platelet lysat ctrl
B
clinical score at end of exp.
bmMSC-EV unstim
bmMSC-EV primed
PBS ctrl
C
bmMSC-EV unstim
bmMSC-EV primed
PBS ctrl
p<0.0001
clinical score
time post IIR [d]
BMT
Treatment
†5/6
0.682
0.54
0.416
D
FoxP3+ [%] of CD4+
bmMSC-EV unstim
bmMSC-EV primed
PBS ctrl
Donor BL/6

Supplement

Figure S1. **Flow cytometry gating strategies**. (A) Gating MSC immunoregulatory marker. From left to right: gating in forward scatters for single cells excluding duplets; gating on MSC population by in forward and side scatter; gating on viable cells, excluding cells stained with viability dye 506; Histograms of PD-L1, PD-L2 and ICAM-1 of viable cells. Percentages of total events as indicated.
(B) Gating strategy for the detection of Tregs. Representative Tregs gating on lymphocytes of $CD3^+$ Tregs induction assay, shown is medium ctrl after 3d. Lymphocyte population was gated according to forward scatter (FSC) characteristics and side scatter (SSC). Gating on viable cells with viability dye by excluding cells stained with viability dye 506. Gating $CD4^+$ T cells of viable lymphocytes and further separation in $CD25^+$, $CD127^{dim}$, $CD4^+$ and intracellular FoxP3 T Cells. Final gating Treg gating on $CD4^+$ $CD25^+$ $CD127^{dim}$ FoxP3 positive population of $CD4^+$ T cells.

Figure S2. **Analysis of extracellular vesicles of stimulated vs. unstimulated MSCs used in murine GvHD models.** Exemplary analysis of MSC-EVs used in figure 5 and figure 6 is shown. Analysis and characterization were also performed with similar results for all other NM-MSC-EVs and BM-MSC-EVs fractions presented in this manuscript. (A and D) TEM image of final EV fraction after precipitation, scale bar: 100 nm. (A) Unstimulated MSC-EVs and (D) Stimulated MSC-EVs. (B-E) NTA analysis of final EV fraction after precipitation, particle concentration (particles/ml) and mean diameter (nm) as indicated. (B) EV fraction of unstimulated MSC-EV and (C) EV fraction of stimulated MSC-EVs. (D) Western blot characterization of EV marker proteins in MSCs supernatant (SN) and its corresponding MSC-EVs (EV) fraction for the expression of HSP70, PD-L1, Flotillin-1, CD81 and CD9. Protein concentration was determined with Pierce BCA protein assay kit; 10 µg were applied on SDS gel for western blotting.

Figure S3: Figure S3: Swiss role analysis of GvHD model. Comparison of colon section of group without GvHD (no GvHD), PBS treated group, unstimulated bmMSC-EV preparations treated or triple cytokine primed bmMSC-EVs preparations treated group, shown in Figure 6 of the manuscript.

Figure S1

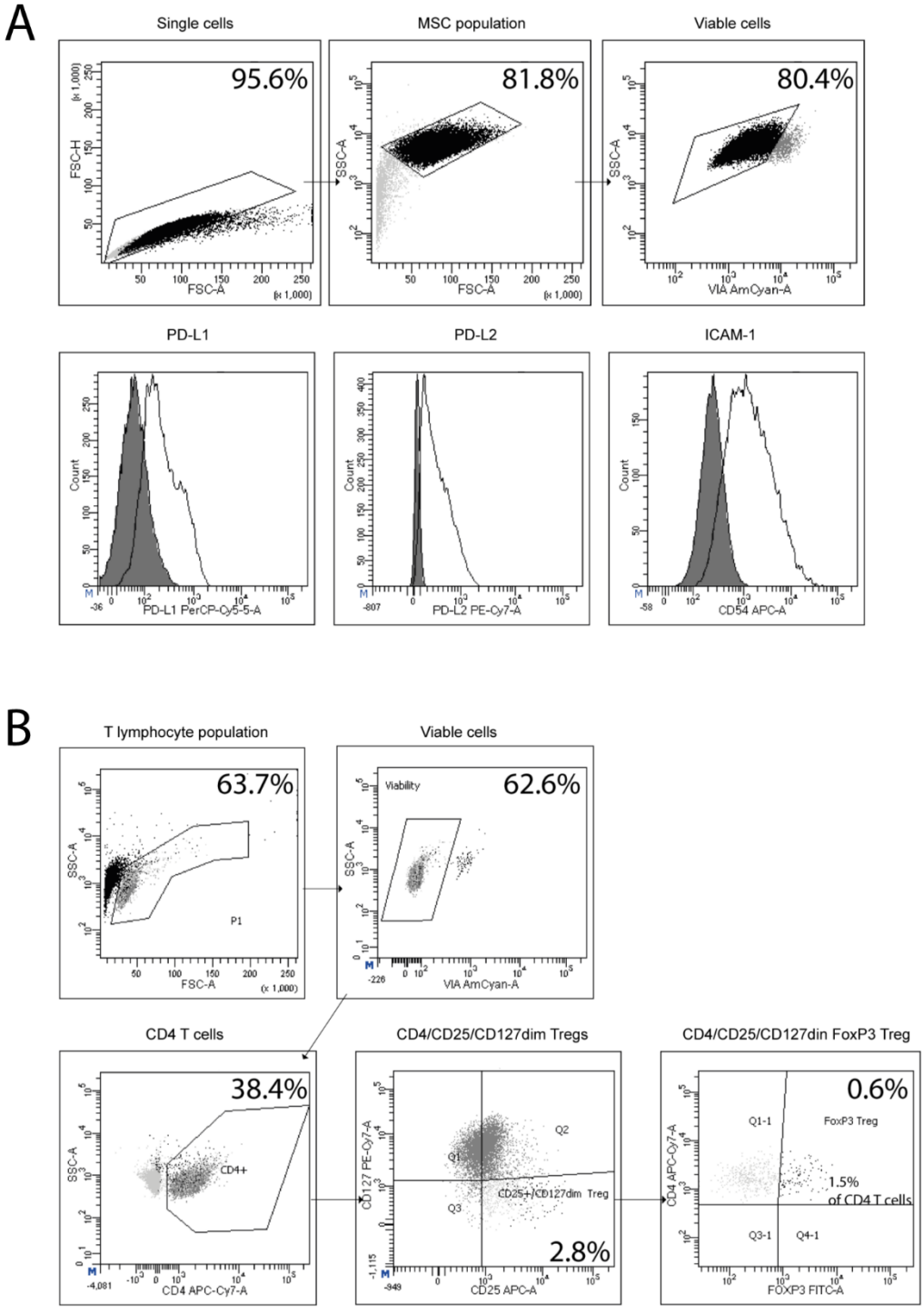
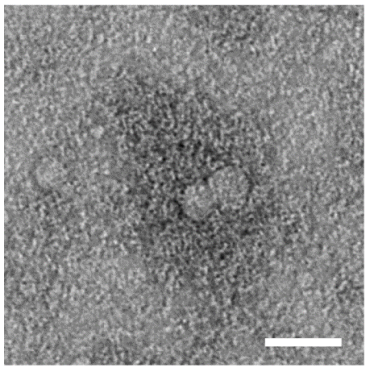

A
Single cells
95.6%
FSC-H
FSC-A
MSC population
81.8%
SSC-A
FSC-A
Viable cells
80.4%
SSC-A
VIA AmCyan-A
PD-L1
Count
PD-L1 PerCP-Cy5-5-A
PD-L2
Count
PD-L2 PE-Cy7-A
ICAM-1
Count
CD54 APC-A
B
T lymphocyte population
63.7%
SSC-A
P1
FSC-A
Viable cells
Viability
62.6%
SSC-A
VIA AmCyan-A
CD4 T cells
38.4%
SSC-A
CD4+
CD4 APC-Cy7-A
CD4/CD25/CD127dim Tregs
Q2
Q1
CD25+/CD127dim Treg
Q3
2.8%
CD127 PE-Cy7-A
CD25 APC-A
CD4/CD25/CD127din FoxP3 Treg
0.6%
Q1-1
FoxP3 Treg
1.5%
of CD4 T cells
Q3-1
Q4-1
CD4 APC-Cy7-A
FOXP3 FITC-A

Figure S2

A



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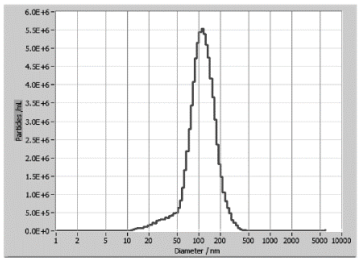
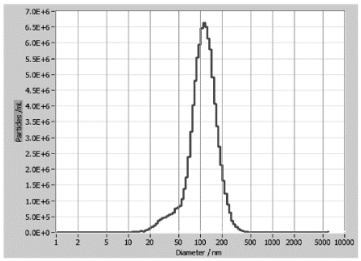


C



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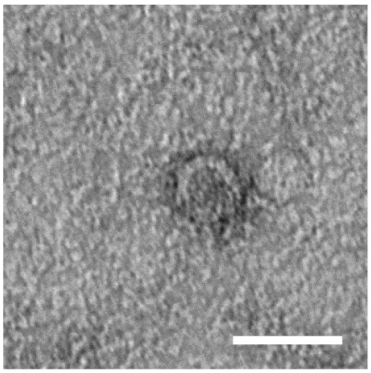


E

Condition	Cytokine-stimulated	Unstimulated
Particle concentration from NTA	1.4 x 10^{11} particles/cm^3	1.7 x 10^{11} particles/cm^3
Average Particle size (X50) from NTA	107,9 nm	106.3 nm

F

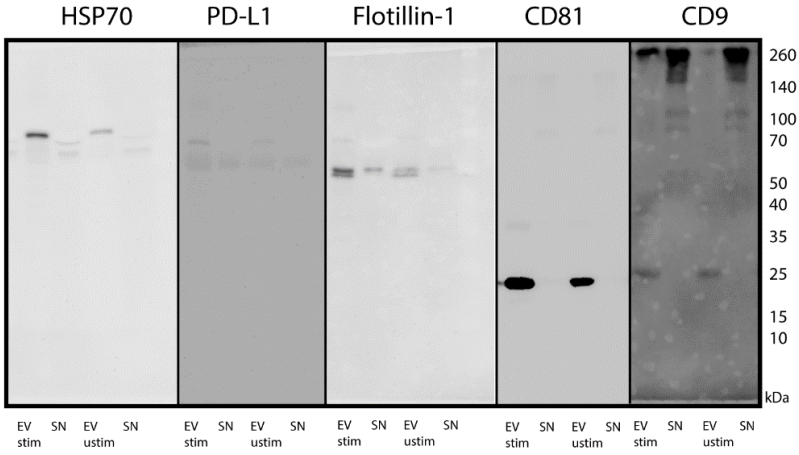


Figure S3

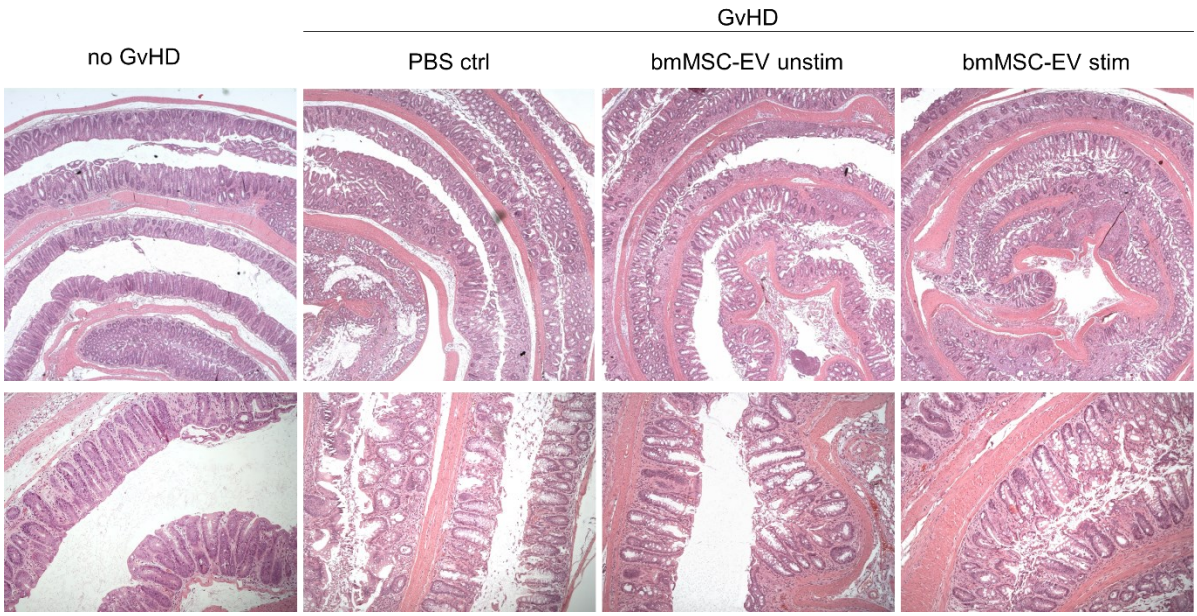


Table S1
Clinical scoring system

Parameter	Clinical Score 0-2 for each parameter For dead/sacrificed animals the clinical score was set at 10.	
Weight loss	0	0% - 10%
	0.5	10% - 15%
	1.0	15% - 20%
	1.5	20% - 25%
	2.0	25% - 30%
Fur	0.5	First Indication of less grooming
	1.0	Fussy fur
	1.5	Skin visible on nose and inner femoral fur
	2.0	Total fussy fur and hair loss

Activity	0.5	Less curious
	1.0	Reduced activity
	1.5	No flight reflex
	2.0	Apathetic
Posture	0.5	First indication of hunching
	1.0	Hunching while sitting
	1.5	Hunching while running
	2.0	Barely able to reach for top chow
Skin	0.5	First indication of dandruffs
	1.0	Clear indication of dandruffs
	1.5	Various dandruffs and small scabs
	2.0	Various dandruffs and large scabs

For the clinical scoring the parameters weight, fur, activity, posture and skin were assessed and scored as indicated in the table. Scores for each parameter were added to give the total score (0-10) for an individual mouse. After death/sacrifice a score of “10” was always assigned.

6. Supplement

Supplemental Report of Proteomic Profiles in MSCs Following Multi-Cytokine Priming

Alexander M. Hackel [ENT Research, University Hospital Essen, Essen, Germany]

Dr. Fouzi El Magraoui [ISAS Institute, Dortmund, Germany]

Prof. Dr. rer. nat. Sven Brandau [ENT Research, University Hospital Essen, Essen, Germany]

Table of Contents

6.1 Introduction

Mesenchymal stem/stromal cells (MSCs) are multipotent cells recognized for their immune-regulatory and regenerative functions (Song et al., 2020). These attributes make MSCs promising candidates for cell-based therapies, and are frequently used in clinical trials to address various diseases, including inflammatory and autoimmune disorders (Jovic et al., 2022).

Building upon previous studies (Hemeda et al., 2010;Hackel et al., 2021;Hackel et al., 2023), this work has employed mass spectrometry proteomics to enable a comprehensive dissection of the molecular changes in response of MSC to different cytokine priming conditions. In recent studies and our own research, it has been observed that the functional properties of MSCs are influenced by various cytokines (Hemeda et al., 2014;Andrews et al., 2022). Notably, an enhanced immunomodulatory response of MSCs has been described through priming with TNF-α, IFN-γ, and IL-1β, advancing the well-established priming with TNF-α and IFN-γ. This has been linked to increased neutrophil recruitment and improved therapeutic efficacy in graft-versus-host disease (Hackel et al., 2021;Hackel et al., 2023).

This study is the first to conduct an extensive comparison of the proteomic profiles of MSCs under diverse cytokine priming conditions. To test the effects of multi-cytokine priming on MSCs, we performed proteomic analysis of MSCs in collaboration with the ISAS Institute (Dortmund, Germany). We analysed unstimulated MSCs, MSCs primed with IL-1β alone and with combined priming with TNF-α and IFN-γ or TNF-α, IFN-γ and IL-1β.

6.2 Aim of the study

The primary objective of this study is to assess the variations in the proteome profile of unstimulated MSCs and after the cytokine priming conditions: IL-1β alone, TNF-α + IFN-γ (dual), TNF-α + IFN-γ + IL-1β (triple). Particularly, the study aims to elucidate the distinct immune responses of MSCs between the conventional dual cytokine priming of MSCs to the triple cytokine priming, suggested to be more efficient by our previous studies. Analysing the significant disparities in cellular responses to these specific cytokine priming conditions may potentially guide future therapeutic strategies and enhance our understanding of MSC biology.

6.3 Material & Methods

6.3.1 Cell Culture and Cytokine Priming

Primary human nasal mucosa mesenchymal stromal cells further referred to as MSCs, were isolated from three healthy donors. The cells were maintained in dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin-

streptomycin, and 1% L-glutamine. Cultures were maintained at 37°C in a humidified incubator with 5% CO_2. For cytokine priming, one million MSCs were treated with IL-1β (10 ng/mL), TNF-α (1000 U), and IFN-γ (1000 U) either singularly or dual and triple combinations as stated.

6.3.2 Samples and Protein Extraction

Following cytokine priming, MSCs were washed twice with phosphate-buffered saline (PBS) and lysed in CHAPS Lysis Buffer containing 1% CHAPS, 40 mM Tris, and 150 mM sodium chloride. Lysates were incubated on ice for 30 minutes and vortexed, followed by centrifugation at 14,000 g for 15 minutes at 4°C. The supernatant was collected and stored at -20°C for further proceedings.

6.3.3 Protein Mass Spectrometry Analysis

Liquid chromatography mass spectrometry (LC-MS/MS) analysis for protein identification and quantification were conducted at the ISAS Institute (Dortmund, Germany).

6.3.4 Data Acquisition and Analysis

Raw MS data were processed using MaxQuant software for protein identification and label-free quantification. Proteins were defined as differential with an adjusted p-value < 0.05 and $-1.5 < \text{logFC} < 1.5$. Log ratios of signal intensities of sample were utilized for clustering analyses and plotting individual proteins.

6.3.5 Statistical Analysis

All statistical analyses were carried out using the R software environment for statistical computing. Protein expression values were normalized, and missing values were imputed using the k-Nearest neighbour algorithm. Differential expression analysis between sample groups was performed using the limma package. Cluster analysis was performed using hierarchical clustering with Euclidean distance and average linkage.

6.3.6 Visualization

Principal Component Analysis (PCA) was used to visualize the overall variance in the dataset and to identify potential outliers. Heatmaps were generated to visualize the expression patterns of differentially expressed proteins across samples. Both PCA and heatmap visualizations were generated using R software (ggplot2 package).

6.3.7 Missing Value Analysis

The missing value plot was constructed to discern patterns associated with missing data in the proteomic dataset. This visualization was bifurcated into two distinct panels. Top Panel: The density plot showcased the log2 intensity values of proteins. Here, proteins characterised with missing values were delineated in blue, while those devoid of missing values were rendered in

red. Bottom Panel: This segment depicted the cumulative fraction of proteins (represented on the y-axis) in relation to their respective log2 intensity values (x-axis). The colouring scheme was consistent with the top panel, using blue and red lines for proteins with and without missing values, respectively. A key inference drawn from the visualization was that proteins associated with missing values generally possessed lower intensity values compared to their counterparts without missing values. This suggests that these absent proteins predominantly belong to the lower abundance category in the dataset.

6.3.8 Venn Analysis

To identify unique and shared proteins among the different priming conditions, a Venn diagram was generated. Using the Venn diagram package in R, sets were created for each condition based on the list of identified proteins. Overlapping regions in the diagram signify proteins that are common between different conditions, whereas non-overlapping portions represent proteins unique to a specific condition.

6.3.9 Data Imputation and Imputed Heatmap

Given the inherent nature of mass spectrometry data, missing values are a common challenge. To address this, an imputation strategy was employed to fill in missing values, simulating values for undetected proteins. The k-Nearest neighbour (k-NN) algorithm was utilized for this purpose, as it considers the similarity between samples to estimate missing values. Following imputation, a heatmap of the imputed dataset was generated to visualize protein expression patterns across samples. The heatmap package in R was used for this purpose. Clustering was applied to both rows (proteins) and columns (samples) to group proteins and samples with similar expression patterns together. The colour scale represents normalized protein expression values, with a gradient from low (blue) to high (red) expression.

6.3.10 Gene Ontology Analysis of differentially Regulated Proteins

The biological significance of proteins differential regulated proteins MSCs after different cytokine priming conditions and without priming, were assessed by Gene Ontology (GO) analysis. Initially, we used PANTHER classification system (http://pantherdb.org) to perform a GO slim analysis for Biological Processes (BP), inputting proteins with a fold change of 1.5 and an adjusted p-value of 0.05. Subsequently, we employed the Database for Annotation, Visualization, and Integrated Discovery (DAVID; https://david.ncifcrf.gov) for a detailed analysis, focusing on "direct" biological processes and applying stringent criteria, including a significance threshold (ease) of 0.05. Additionally, the interaction networks and functional associations between the differentially regulated proteins were analysed with the STRING database (Search Tool for the Retrieval of Interacting Genes/Proteins). String analysis allows

to visualize and analyse the relationships and interactions among the proteins, offering insights into the potential BPs influenced by cytokine priming.

6.4 Results and Interpretations

Proteomic analysis was conducted on MSCs obtained from three donors at passage four. These cells were cultured in following conditions: unstimulated, primed with IL-1β, with TNF-α + IFN-γ (dual), and with TNF-α + IFN-γ + IL-1β (triple) for 24 hours. The proteomic profiling was performed at the ISAS Institute (Dortmund, Germany) and subsequent data analysis was carried out in our ENT-Research laboratory (University Hospital Essen, Essen. Germany). A detailed list of identified proteins is provided in **(Supplement Table 1-3)**.

6.4.1 Quality control and Data assembling

Critical to the accuracy of proteomic data is the quality control and assessment. In this regard, a missing value heatmap, presented in **(Figure 1 A, C)**, was generated to visualize protein detection across the samples. In this heatmap, each row represents specific proteins with at least one missing value, while columns correspond to the samples. Detected proteins are indicated in black, while absent proteins in specific samples are shown in white. These samples were clustered based on their missing value patterns. Based on the pattern, it can be deduced that the missing values occur at random rather than exhibiting a specific condition or bias. Notably, the missing value pattern of the IL-1β 1 sample, as shown in (**Figure 1A)**, stands out among the other samples. This particular sample had more missing values compared to the rest. Across all 12 samples, while each generally revealed 800 to 900 proteins, only about 300 proteins were consistently identified, suggesting these may constitute a core proteomic profile. The variation in the remaining proteins could offer valuable insights into differential biological processes or responses under the priming conditions.

Analysing the PCA plot, it became evident that the IL-1β 1 sample identified with an abnormal variance compared to the other samples **(Figure 1 B)**. Consequently, based on these findings, the IL-1β 1 sample has been excluded from further data analysis in protein studies. In its absence of this sample, the heatmap displayed a consistent pattern in missing values **(Figure 1 C)** and the samples cluster based on the treatment conditions **(Figure 1 D)**. The proteomic profile of the dual primed MSCs appeared similar to that of the unstimulated MSCs based on the PCA. However, they also exhibited a relatively wide distance from each other within the PCA plot. In the context of PCA, this distance indicated distinct variance and potential differences in their protein expression profiles or underlying biological processes.

Figure 1

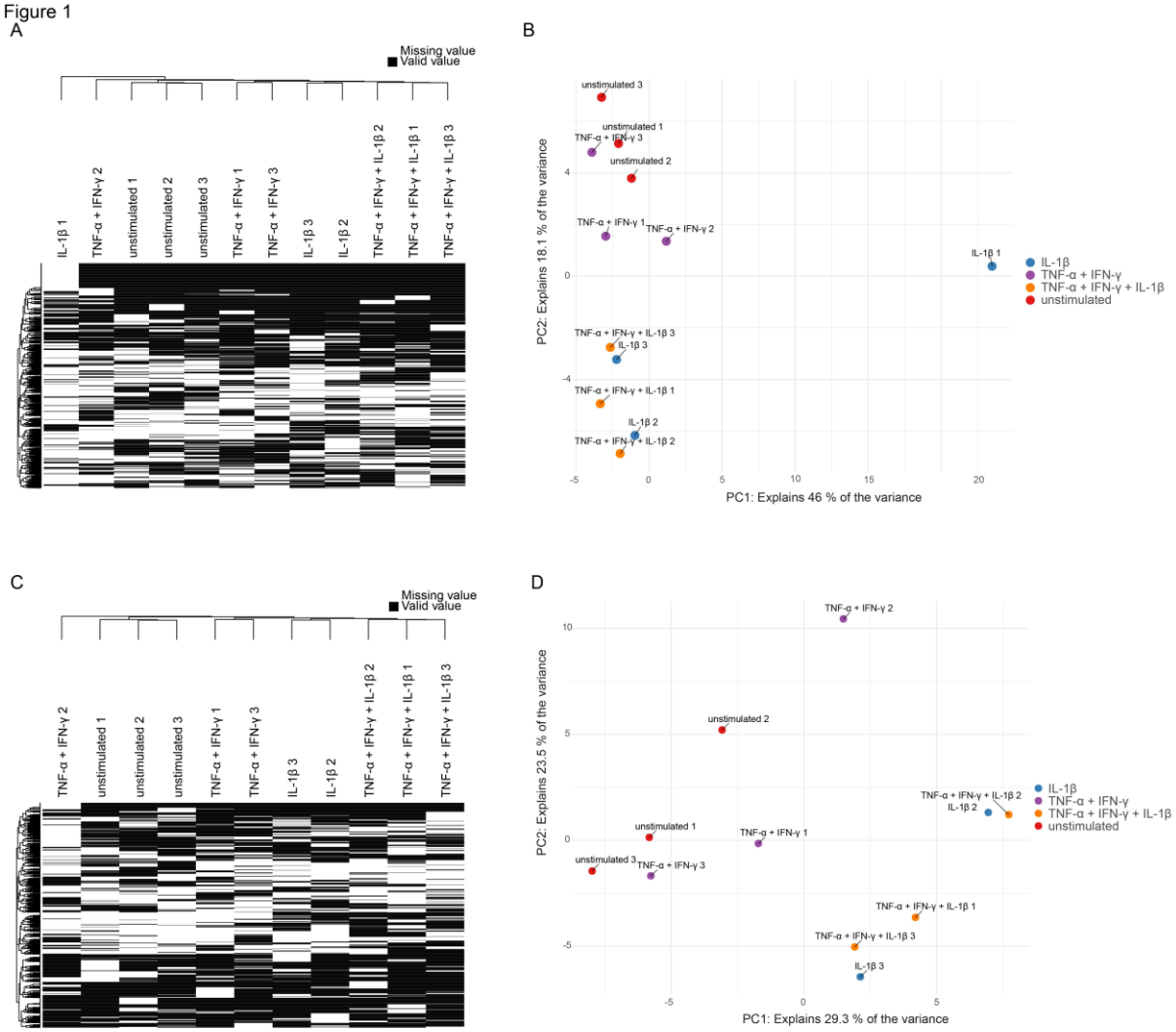


Figure 1: Assessment of Proteomic Data Quality and Variance. **A**: Missing value heatmap visualizes protein detection across samples, with each row representing proteins with at least one missing value and columns corresponding to samples. Detected proteins are shown in black, while absent proteins are in white. The samples are clustered based on missing value patterns, suggesting random occurrence of missing values. The IL-1β 1 sample displays a distinctive pattern with a higher number of missing values, indicating unique factors influencing protein absence. **B**: The PCA plot reveals abnormal variance in the IL-1β 1 sample compared to others. **C, D**: After excluding the IL-1β 1 sample, the heatmap (C) shows consistent missing value patterns, and samples cluster based on treatment conditions (D).

6.4.2 Characteristic Clustering of Triple and IL-1β Primed MSCs

MSCs primed with a combination of cytokines, including triple, dual, and single priming with IL-1β, were analysed against unstimulated MSCs for baseline control. This approach enabled us to discern the differential proteome responses of the respective cytokine priming and particularly to evaluate the contributory effect of IL-1β in the context of triple priming. The differential expressed proteins (with a fold change ≥ 1.5 and $p \leq 0.05$) of the respective cytokine priming conditions compared to the unstimulated controls are shown in **Figure 2**. Triple and single IL-1β priming demonstrated the highest number of differential expressed proteins

(Figure 2 A-C). The Venn diagram shows the differential and common proteins expressed in MSCs across the different priming conditions **(Figure 2 B)**. The triple primed MSCs (TNF-α + IFN-γ + IL-1β vs unstimulated) emerge as the most distinct. This condition uniquely expresses six proteins. The IL-1β compared to unstimulated condition stands out with three proteins. Lastly, for dual priming only two proteins were distinct from the unstimulated MSCs. Furthermore, the IL-1β and dual priming had two shared proteins. A substantial overlap is discerned between IL-1β and triple priming, with nine proteins in common. Interestingly, there are two shared entities spanning across all three conditions, and similarly, none between the Double and Triple conditions. Overall, the Venn diagram effectively elucidates the intricate relationships of proteins among the experimental conditions **(Figure 2 B)**.

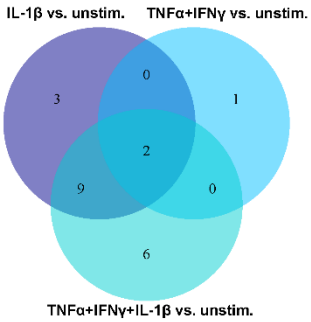
The heatmap illustrates a similarity in protein expression profiles, with one cluster showed a resemblance between IL-1β and triple priming, distinguished from the cluster comprising dual and unstimulated conditions **(Figure 2 C**). The proteomic profiles of MSCs primed with IL-1β and the triple combination showed distinct responses. This pattern underscores the unique proteomic response elicited by IL-1β, particularly when part of the triple priming response. Notably, in these conditions, key proteins such as phosphoribosyltransferase (NAMPT) and signal transducer and activator of transcription 1, 3 (STAT1 and SATAT3), which are critical for immunological functions, were markedly upregulated. NAMPT plays a dual role in immunity, intracellularly influencing cellular metabolism and survival, as well as mediating pro-inflammatory cytokines in monocytes and macrophages (Audrito et al., 2020). Elevated in chronic inflammatory diseases by mediating T cell activation and differentiation into subsets like Tregs and Th17 cells (Navarro et al., 2022). In the context of immune suppression mediated by myeloid-derived suppressor cells (MDSCs), NAMPT can suppress T Cells and support tumor growth (Audrito et al., 2019). IL-1β appears to be a critical factor in inducing these specific proteomic changes. This suggests that IL-1β plays a pivotal role in shaping the immune response of MSCs by triple priming, potentially through pathways involving proteins like NAMPT, STAT1 and STAT3. In contrast, collagen type I alpha 1 chain (COL1A1) and collagen type I alpha 2 chain (COL1A2) exhibit consistent downregulation across the IL-1β and triple conditions but not in the dual and unstimulated conditions. The downregulation of COL1A1 and COL1A2 could signify a potential reduction in the fibrotic activity of MSCs.

Figure 2

A

TNFα+IFNγ+IL-1β vs. unstim.	TNFα+IFNγ vs. unstim.	IL-1β vs. unstim.	IL-1β vs. unstim.: TNFα+IFNγ+IL-1β vs. unstim.	IL-1β vs. unstim.:TNFα+IFNγ vs. unstim.: TNFα+IFNγ+IL-1β vs. unstim.
SERPINB2	PHB2	OPTN	WARS	SOD2
APM2		IFITM3	LAP3	COL1A2
LUC7L3		GRB2	STAT1	
AKAP12			HLA-A	
EIF2AK2			SAMHD1	
KARS			STAT3	
			COL1A1	
			PSME1	
			NAMPT	

B



C

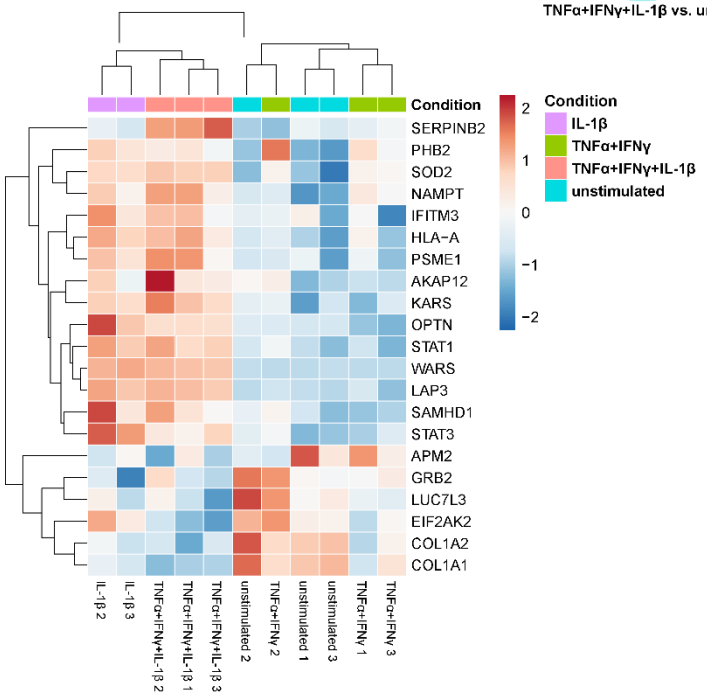


Figure 2: Characteristic Clustering of Triple and IL-1β Primed MSCs. **A-C:** The number of differentially expressed proteins (fold change ≥1.5, adj. $p \leq 0.05$) is depicted, with the highest counts observed in triple and single cytokine priming conditions. **B**: The Venn diagram delineates the unique and shared proteins among the different priming conditions. **C**: The heatmap shows the protein expression profiles, clustering single cytokine and triple priming together, distinct from dual and unstimulated conditions. Certain proteins are consistently upregulated in cytokine primed conditions, suggesting enhanced immunomodulatory functions, while others are downregulated, indicating a potential reduction in fibrotic activity.

6.4.3 Gene Ontology Analysis Implicate Enhanced Tissue Regeneration and Immune Modulation through Triple Cytokine Priming

In our proteomic analysis of triple primed MSCs compared to unstimulated MSCs showed the most differential abundance of proteins **(Supplement Table 1-3)**. We assessed the gene ontology (GO) terms connected to the identified proteins with a fold change of 1.5 and an adjusted p-value of 0.05 **(Figure 3 A-D)**.

For the upregulated proteins of triple primed MSCs compared to unstimulated MSCs. The GO slim analysis for biological processes (BP) by PANTHER revealed a significant involvement 40.7% in "cellular processes" (GO:0009987), associated with 11 proteins, indicating the cellular adaptations and responses post cytokine priming. Similarly, proteins involved in "metabolic processes" (GO:0008152), making up 25.9% with seven proteins, highlight the metabolic shifts and the fundamental pathways activated in response to cytokines. Additionally, 14.8% proteins ascribed to "biological regulation" (GO:0065007) demonstrate the regulatory mechanisms MSCs undergo post cytokine exposure. Lastly, "Response to stimulus" (GO:0050896) with

three proteins, accounting for 11.1%, showcases the MSCs' ability to perceive and react to the external cytokine stimulus **(Figure 3 A)**. In sum, this analysis implicates processes MSCs undergo in response to cytokine priming, from cellular maintenance to defence, ensuring their functionality in tissue regeneration and immune modulation. Together, this highlights the multiple processes MSCs are involved, from cell maintenance to defence mechanisms, also involving regenerative and immunomodulatory roles.

For a more precise suggestions of biological processes, we applied our results to the Database for Annotation, Visualization, and Integrated Discovery (DAVID), an online-tool offered by the National Institutes of Health (NIH). By focusing on “direct” biological processes within the DAVID database, we implemented stringent criteria, adding to factors like fold change and adjusted p-value like mentioned. Further, we employed a significance threshold (ease) of 0.05. Such rigorous parameters ensured a minimization of generic associations, elevating the reliability of our findings **(Figure 3 B)**.

Consequently, our analysis identified nine significant BP direct GO terms. The stringent DAVID GO analysis, however, showed sparse protein associations with these BPs, indicating a possibility of exaggerated interpretations of the BP results. Remarkably, proteins like STAT1, STAT3, and NAMPT, integral to immunoregulation, were found to play pivotal roles in these BPs. Central to our findings were processes associated with the regulation of cell morphogenesis and signal transduction. The processes "response to hydrogen peroxide" ($p=0.0000063$) and "response to electrical stimulus" ($p=0.00031$) were represented by proteins such as SOD2 overlapping transcript 1 (SOD2-OT1), STAT3, SOD2, and tryptophanyl-tRNA synthetase 1 (WARS1). Further, we identified processes associated with "positive regulation of nitric-oxide synthase biosynthetic process" ($p=0.000096$), "protein homotetramerization" ($p=0.0011$), "response to lipopolysaccharide" ($p=0.0065$), and "positive regulation of cell migration" ($p=0.0013$). Predominantly involved in these mechanisms were proteins such as A-kinase anchoring protein 12 (AKAP12), NAMPT, STAT1, and STAT3 analysis. In line with the GO analysis, predicted protein-protein interactions by the STRING database showed that, among others, the immunoregulatory proteins STAT1, STAT3 and NAMPT were at the centre of the string connections **(Figure 3 C)**. This central positioning suggests that these proteins may play a pivotal role in the observed cellular response. Their interconnection underscores their potential significance in the signalling pathways or protein complexes. The combined analyses highlight the diverse adaptations MSCs make at the molecular level in response to triple cytokine priming, underscoring their potential for tissue regeneration and immune modulation.

Further studies on these central proteins could provide insights into their cooperative roles and importance in cellular processes. Specifically, for the downregulated proteins of triple primed MSCs, only COL1A1 and COL1A2 are associated with cellular processes and metabolic processes by PANTHER GO slim **(Figure 3 D)**. Analysis by David BP direct revealed for example associations with "skin morphogenesis" (p=0.027), "blood vessel development" (p=0.0098), "cellular response to amino acid stimulus (p= 0.013), "collagen fibril organization" **(Figure 3 E)**. Matching these findings, STRING analysis illustrates a protein interaction network with COL1A1 and COL1A2 **(Figure 3 F)**. It is noteworthy that merely two proteins from the same family are linked to these GO terms, suggesting that these results are indicative rather than conclusive and require substantiation through comprehensive experiments.

Figure 3

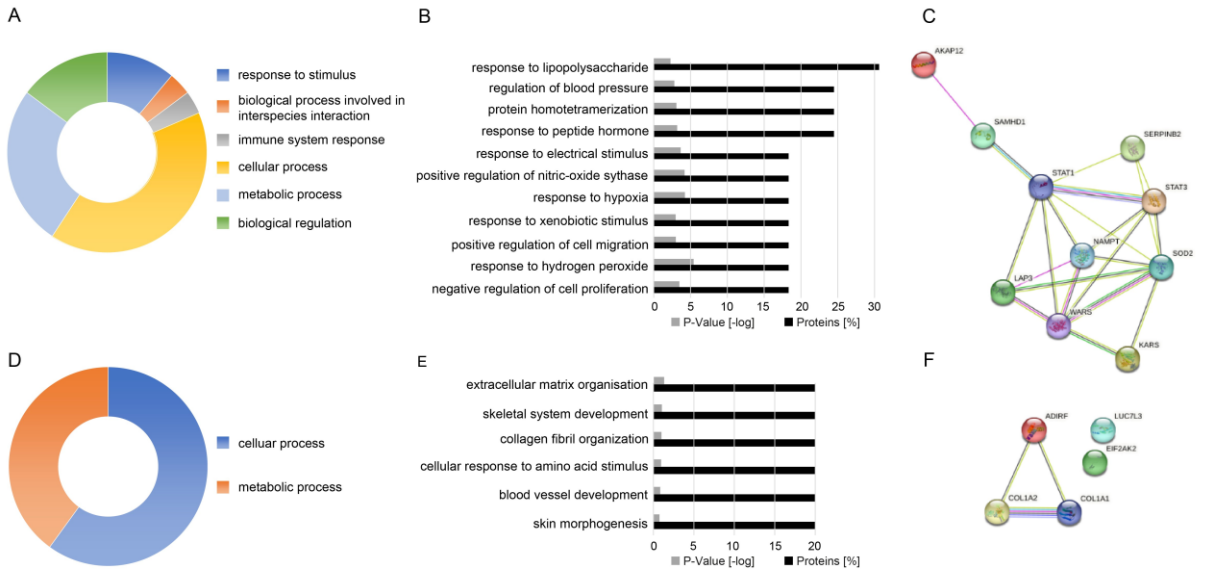


Figure 3: GOs of tripled primed MSCs suggests involvement in tissue regeneration and immune modulation **A**: PANTHER GO slim analysis of upregulated proteins in triple primed MSCs (n=3) against unstimulated controls (n=3). **B**: DAVID GO analysis comparison of the "direct" biological processes (ease: 0.05) between triple primed and unstimulated MSCs. **C and F**: STRING database predictions compare protein-protein interactions among triple primed MSCs. **D-E**: PANTHER GO slim and DAVID BP direct analyses compares downregulated proteins in triple primed MSCs to unstimulated controls.

6.4.4 Triple Priming Augments MSCs' Immunoregulatory Activity Compared to Dual Cytokine Priming

Upon comparing the protein expression profiles of triple and IL1beta primed mesenchymal stem cells, it was observed that only SERPINB2 demonstrated a significant upregulation, as detailed **(Supplement Table 2)**. This finding underlines the need for careful interpretation due to the minimal differences between the groups. Nevertheless, SERPINB2 is integral to the immunoregulation of mesenchymal stem cells, influencing inflammatory responses by

modulating pro-inflammatory cytokines and aiding in tissue remodelling through the regulation of matrix metalloproteinases. BPs indicative for downregulated proteins identified by DAVID GO direct were "negative regulation of viral genome replication" (p=0.0092), "response to virus" at (p=0.022), "translation" (p=0.045), and "defense response to virus" (p=0.048), **(Figure 4 A)**.

The analysis of upregulated proteins of MSCs primed with triple compared to dual cytokine priming **(Supplement Table 3)**, identified several key biological processes through PANTHER BP GO-Slim analysis **(Figure 4 B)**. Among the most significantly affected were "cellular processes" (46.7%) and the "metabolic process" (40%). Additionally, processes integral to the "immune system" (13.3%) and the "response to stimulus" (20%) were impacted. The data suggests that MSCs may be fine-tuning their response mechanisms under triple cytokine priming, leading to a stronger modulation in their immunoregulatory capabilities compared to dual cytokine priming. The analysis of direct BP by DAVID identified "defense response to viruses" (p=0.012), as indicated by the upregulation of proteins such as SAM and HD domain containing deoxynucleoside triphosphate triphosphohydrolase 1 (SAMHD1), interferon-induced transmembrane protein 3 (IFITM3) and STAT1 **(Figure 4 C)**. Other processes identified include "response to interferon-beta" (p=0.0079), "response to interferon-gamma" (p=0.019), "interferon-gamma-mediated signalling pathway" (p=0.02) and the "regulation of I-kappaB kinase/NF-kappaB signalling" (p=0.04). Interestingly, these BPs highlight upregulated and central role STAT1 **(Figure 4D)**. In conclusion, IL-1β has been demonstrated to be a critical mediator in the immune response elicited by triple cytokine priming in MSCs. This effect could be facilitated by pathways involving NF-κB and STATs in a biphasic response to the triple priming, as discussed by Hackel et al. (2021).

Figure 4

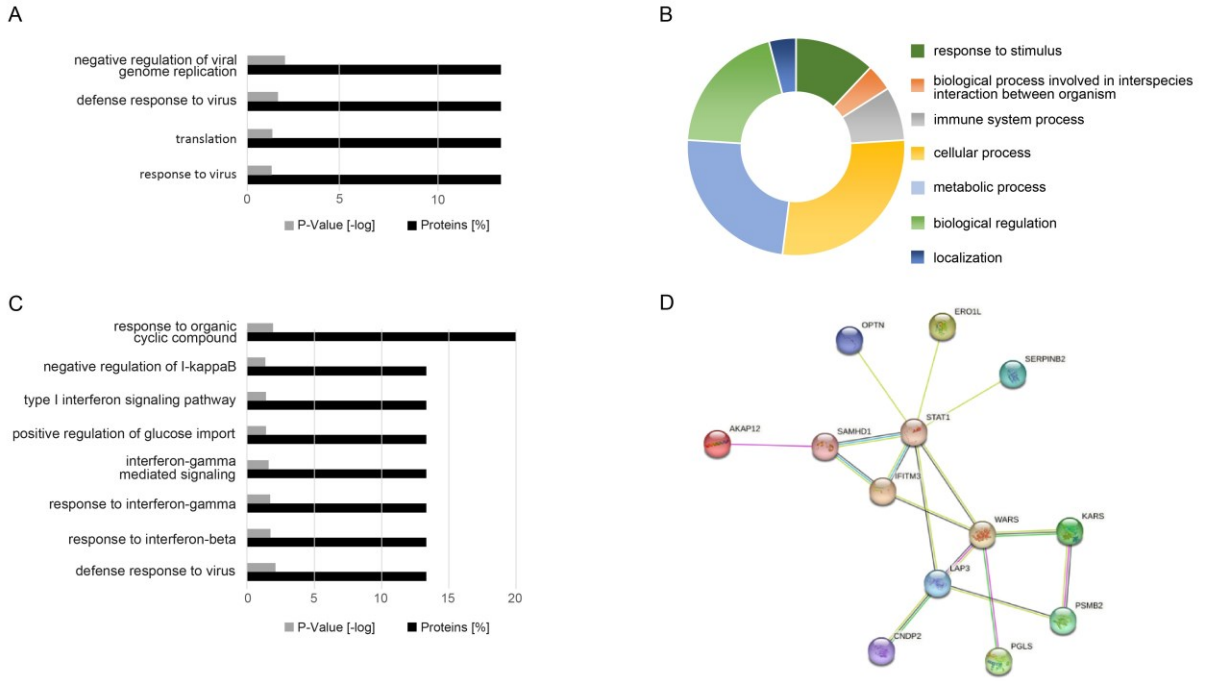


Figure 4: Enhanced immunoregulatory activity in MSCs indicated by triple priming compared to dual priming. Comparative analysis proteomic responses of MSCs under triple cytokine priming (n=3) versus IL-1β (n=2) or dual cytokine priming (n=3). **A**: Minor associations in DAVID GO analysis of BP "direct" (ease: 0.05) of downregulation of proteins in triple primed MSCs compared to IL-1β primed MSCs. **B**: PANTHER BP GO-Slim analysis and **C**: DAVID GO BP "direct" analysis of upregulated proteins in MSCs under triple priming compared dual priming. **D**: STRING database predictions for protein-protein interactions for significant upregulated proteins in triple primed compared to dual primed MSCs.

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6.6 Supplemental Tables

Supplement Table 1: Significant differentially regulated proteins of triple primed MSCs (n=3) compared to unstimulated MSCs (n=3)

Proteins	logFC	adj. p-value	Uniprot ID
WARS	4.178	8.298E-14	P23381
STAT1	4.072	6.286E-12	P42224
SOD2	3.759	8.298E-14	P04179
NAMPT	3.085	6.824E-04	P43490
LAP3	2.888	8.298E-14	P28838
HLA-A	2.664	1.296E-09	P01892
SERPINB2	2.617	1.835E-05	P05120
SAMHD1	1.983	2.359E-04	Q9Y3Z3
STAT3	1.233	2.238E-02	P40763
PSME1	1.225	3.010E-03	Q06323
AKAP12	1.046	3.357E-02	Q02952
KARS	0.983	4.890E-02	Q15046
EIF2AK2	-1.307	4.559E-02	P19525
APM2	-1.375	2.272E-04	Q15847
LUC7L3	-1.588	7.437E-03	O95232
COL1A2	-2.701	1.372E-07	P08123
COL1A1	-3.437	2.712E-06	P02452

Supplement Table 2: Significant differentially regulated proteins of triple primed MSCs (n=3) compared to IL-1β primed MSCs (n=2)

Proteins	logFC	adj. p-value	Uniprot ID
SERPINB2	2.349	8.08E-05	P05120
MX1	-0.972	0.002	P20591
APM2	-0.979	0.001	Q15847
SARS	-1.221	0.001	P49591
EIF2AK2	-1.326	0.001	P19525
CDC42EP1	-1.341	0.001	Q00587

Supplement Table 3: Significant differentially regulated proteins of triple primed MSCs (n=3) compared to dual primed MSCs (n=3)

Proteins	logFC	adj. p-value	Uniprot ID
WARS	4.296	1.227E-13	P23381
STAT1	3.690	2.454E-12	P42224
LAP3	2.857	1.227E-13	P28838
SERPINB2	2.554	1.841E-06	P05120
SAMHD1	1.872	7.997E-05	Q9Y3Z3
HLA-A	1.839	1.949E-05	P05534
OPTN	1.529	9.796E-05	Q96CV9
PSMB2	1.119	0.043	P49721
PSME1	1.081	0.004	Q06323
PGLS	1.081	0.034	M0R1L2
IFITM3	1.062	0.008	Q01628
ERO1L	1.044	0.007	Q96HE7
CNDP2	1.007	0.016	Q96KP4
AKAP12	0.889	0.045	Q02952
KARS	0.882	0.041	Q15046
BCL2L13	-0.962	0.048	A0A087WTL4
APM2	-1.069	0.004	Q15847
NENF	-1.109	0.046	Q9UMX5
MCTS1	-1.114	0.036	Q9ULC4
GOT1	-1.218	0.023	P17174
COL1A1	-1.814	0.045	P02452

7. Resume

The cumulative studies deepen the understanding of MSCs' involvement in immunity by specifically examine their immune responses in a pro-inflammatory environment and their subsequent interaction with other immune cells. In this regard, the studies elucidate the immunoregulatory characteristics of MSCs and their EVs after multi-cytokine priming.

MSCs, originally identified in bone marrow and subsequently isolated from various tissues including adipose tissue, connective tissue, and umbilical cord, are characterized by their plastic adherence, specific surface markers, and the absence of certain endothelial and hematopoietic cell surface antigens. Their ability to differentiate into diverse lineages such as chondrocytes, osteoblasts, and adipocytes, highlights their importance as stromal progenitor cells numerous tissues (Pittenger et al., 1999;Jakob et al., 2010).

Moreover, MSCs play a pivotal role in immunity, converting immune signals into immunoregulatory activity and exhibiting profound immunomodulatory functions. These functions are particularly evident during inflammatory processes, where MSCs respond to pathogenic and cytokine stimuli, modulating immune responses through both direct cell-cell contact and paracrine mechanisms (Hemeda et al., 2010;Brandau et al., 2014; Dumitru et al., 2014).

Over the past decade, the therapeutic potential of MSCs have gained profound attention, particularly in their capacity to modulate immunity and facilitate tissue repair, as evidenced by numerous clinical studies (Zhou et al., 2021;Galderisi et al., 2022). Research has also elucidated the effects of cytokine priming on MSCs, aiming to mimic inflammatory environments and to enhance their therapeutic efficiency (Noronha et al., 2019;Lim et al., 2021). The exposure to proinflammatory cytokines like TNF-α, IFN-γ or IL-1β can significantly alter the immunoregulatory functions of MSCs, leading to upregulation of various immunoregulatory proteins that are crucial in both, innate and adaptive immunity (Krampera et al., 2006; Hemeda et al., 2010;Yu et al., 2019). However, the response of MSCs to these complex cytokine environments remains with limited understanding, primarily due to the focus on simpler single or dual cytokine stimulations in past studies.

In this context, we focused on understanding how MSCs convert cytokine signals into cellular immunoregulation. We investigated the biological and kinetic responses of MSCs to a combination of three key pro-inflammatory cytokines, previously known to modulate MSC functions: TNF-α, IFN-γ and IL-1β (Hackel et al., 2021). Within the experiments we assessed the impact of these cytokines on MSCs' cytokine secretion patterns, particularly focusing on

the activation and recruitment of neutrophils. Our findings revealed that the combination of TNF-α and IFN-γ, already established for its immunoregulatory effects, was significantly augmented by the addition of IL-1β (referred to as triple priming). We found a molecular feedback loop over the IFN-γ/NF-kB pathway, which resulted in a heightened release of IL-8 and, consequently, an augmented recruitment of PMNs. Interestingly, our proteomic study strengthened these findings by demonstrating higher protein abundance of STAT family members after triple priming of MSCs **(Supplement)**. Recent studies emphasized PMNs to be pivotal in maintaining tissue homeostasis by efficiently removing damaged cells (Peiseler and Kubes, 2019;Oved et al., 2021). Advancing the knowledge of MSC and PMN interactions in inflammatory settings could lead to therapeutic strategies, potentially minimizing systemic trauma complications and aiding in the treatment of systemic inflammatory response syndrome.

In the second study, we successfully employed our results to amplify the therapeutic efficiency of MSCs and their EVs in a murine GvHD model (Hackel et al., 2023). We found that muti-cytokine primed MSCs and EVs displayed pronounced immunosuppressive effects on alloreactive T cells and mediated an amplified induction of regulatory T cells, in a PD-1 dependent manner. These findings gain importance considering the clinical study of Kordelas et al., which describes PD-1 as a critical predictor for clinical outcomes in GvHD after HSCT (Kordelas et al., 2021). Thus, underlining the clinical relevance of targeting PD-1 pathways. Proteomic analysis of MSCs, subjected to the defined multi-cytokine priming **(Supplement)**, further substantiated the therapeutically advantageous alterations in MSC biology. This analysis emphasized the downregulation of collagen I and II, which potentially reduce fibrosis and thereby alleviate the severity of GvHD. The established long-term GvHD model further serves as a general model for systemic and chronic inflammatory diseases and provides viable insights to the long-term therapeutic effects of multi-cytokine primed MSCs and EVs (Miceli et al., 2021). Thereby multi-cytokine primed MSCs and their EVs provide implications for chronic inflammatory diseases, in particular connected tissue diseases and vasculopathies (Rozier et al., 2021;Huldani et al., 2022). An important finding from both studies was a distinctive response of MSCs to the defined triple cytokine priming that implied to be independent of the MSCs' origin.

Ultimately, the application of primed MSCs and their derived EVs provides an innovative strategy for modifying patient immune responses in a therapeutically beneficial manner, potentially altering treatment paradigms for numerous chronic inflammatory disorders.

Nevertheless, despite the encouraging results of these studies, it remains imperative to further elucidate the molecular mechanisms by which MSCs exert their immunoregulatory function.

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9. Appendix

Curriculum Vitae

For reasons of data protection, the curriculum vitae is not included in the online dissertation.

Declaration:

In accordance with § 6 (para. 2, clause g) of the Regulations Governing the Doctoral Proceedings of the Faculty of Biology for awarding the doctoral degree Dr. rer. nat., I hereby declare that I represent the field to which the topic „*Optimizing the Immunoregulatory Properties of Human Mesenchymal Stromal Cells and their Extracellular Vesicles by Immunological Priming"* is assigned in research and teaching and that I support the application of (Alexander Maximilian Hackel).

Essen, date _____ Prof. Dr. Sven Brandau _____

Name of the scientific supervisor/member of the University of Duisburg-Essen

Signature of the supervisor/ member of the University of Duisburg-Essen

Declaration:

In accordance with § 7 (para. 2, clause d and f) of the Regulations Governing the Doctoral Proceedings of the Faculty of Biology for awarding the doctoral degree Dr. rer. nat., I hereby declare that I have written the herewith submitted dissertation independently using only the materials listed, and have cited all sources taken over verbatim or in content as such.

Essen, date _____ _____

Signature of the doctoral candidate

Declaration:

In accordance with § 7 (para. 2, clause e and g) of the Regulations Governing the Doctoral Proceedings of the Faculty of Biology for awarding the doctoral degree Dr. rer. nat., I hereby declare that I have undertaken no previous attempts to attain a doctoral degree, that the current work has not been rejected by any other faculty, and that I am submitting the dissertation only in this procedure.

Essen, date _____ _____

Signature of the doctoral candidate

Annex to the Application for Admission to the Doctoral Examination
Documentation of accreditation achieved during the qualifying phase in accordance with § 6a of the Regulations Governing the Doctoral Proceedings of the Faculty of Biology of the University of Duisburg-Essen dated 05.08.2013 (Gazette Year 11, 2013 p. 955/No. 121).

Credit points may be attained by (c.f. Doctoral Proceedings § 6a):	Accreditation attained during the qualifying phase	Credit points (CP)
– Participation in courses for the acquisition of interdisciplinary qualifications (soft skills)		
– Participation in specific modules of the Faculty's Masters programme as doctoral project preparation		
– Conduction of teaching courses or leading study groups		
– Participation at conferences with presentation of own work		
– Other comparable achievements		

Alternatively, the accreditation during the qualifying phase has been achieved within the framework of the following structured doctoral programme (certification attached):

Graduate School of Biomedical Science „BIOME“

The accreditation of 18 credit points during the qualifying phase has been achieved:

Alexander M. Hackel — **Prof. Dr. Seven Brandau**

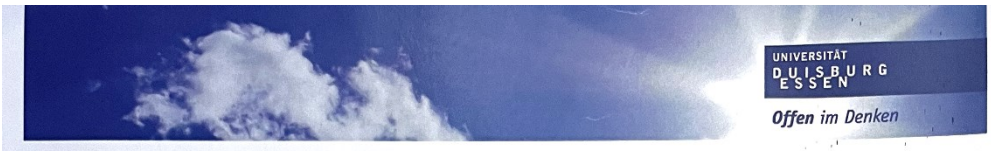
Doctoral Candidate — **Doctoral Supervisor**

Essen, date: ……

……

Signature of the Doctoral Candidate — **Signature of the Doctoral Supervisor**

BIOME Certificate



Graduate School of Biomedical Science

BIOME

GRADUATE SCHOOL OF BIOMEDICAL SCIENCE (BIOME)

CORE: "CELLULAR AND MOLECULAR IMMUNOLOGY"

CERTIFICATE OF PARTICIPATION

This is to certify that **Alexander Hackel** participated in our graduate school on the course "Cellular and Molecular Immunology" from **October 2016 – September 2019.** This school is a collaboration between the Faculty of Medicine and the Faculty of Biology at the University Duisburg-Essen in Germany.

The framework of this course is the fortnightly lecture series where the supervisors present aspects of their area of expertise. The focus of the course is on the fundamentals on immunology, on the immunologic interaction of a tumour and its host, and on transplantation immunology. These topics represent core areas of clinical, translational and basic research at the Faculty of Medicine and in cooperating groups of other faculties. Clinical aspects as well as the underlying cellular and molecular mechanisms are also covered. The curriculum on cellular and molecular mechanisms touches on all cell types of the immune system. Lectures and seminars on molecular immunology focus on classes of receptors, signal transduction pathways and mechanisms of gene regulation, which are pivotal to the function of immune cells. Members of this course also obtain insight into a number of methods of modern immunology, including the imaging of extra- and intracellular events in immune cells, assays analyzing cell-cell interactions, manipulation of immune cells, tissue analysis and finally the use of animal models in immunology.

Also worth mentioning are the annual retreats in Hamminkeln in November 2016, in Xanten in November 2017, and in Cologne in November 2018, where a number of internationally-recognised scientists were invited to speak about their work and graduates like Alexander presented their latest findings in talks and poster sessions.

Additionally, Alexander presented his own research data to a critical peer audience twice during the regular doctoral seminar presentations held directly after each lecture.

Due to the international nature of the graduate school itself and the scientific world in general, the lingua franca of the entire course is English.

Alexander also successfully attended soft skill workshops on biostatistics, research funding for next-generation scientists, and two workshop series on the basics of experimental work in animals.

Furthermore, Alexander attended the IGLD Annual Meetings in Düsseldorf in March 2017 and in Frankfurt in March 2018, gave a talk at the 47th Annual Meeting of the German Society for Immunology (DGFI) in September 2017, as well as being invited to present a poster at the Keystone Symposium on

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UNIVERSITÄT DUISBURG ESSEN

Offen im Denken

Exosomes/Microvesicles: Heterogeneity, Biogenesis, Function and Therapeutic Developments in Breckenridge, Colorado, in June 2018.

Alexander Hackel has proven that as a competent scientist he has no difficulties working within an English-speaking, international environment.

Prof. Dr. Ulf Dittmer
-Chairman-

Publications and Conference Contributions

First Author

Hackel, A., Aksamit, A., Bruderek, K., Lang, S., & Brandau, S. (2021). TNF-α and IL-1β sensitize human MSC for IFN-γ signaling and enhance neutrophil recruitment. Eur J Immunol, 51(2), 319-330. doi:10.1002/eji.201948336

Hackel, A., Vollmer, S., Bruderek, K., Lang, S., & Brandau, S. (2023). Immunological priming of mesenchymal stromal/stem cells and their extracellular vesicles augments their therapeutic benefits in experimental graft-versus-host disease via engagement of PD-1 ligands. Front Immunol, 14, 1078551. doi:10.3389/fimmu.2023.1078551

Second Author

Petri, R. M., Hackel, A., Hahnel, K., Dumitru, C. A., Bruderek, K., Flohe, S. B., . . . Brandau, S. (2017). Activated Tissue-Resident Mesenchymal Stromal Cells Regulate Natural Killer Cell Immune and Tissue-Regenerative Function. Stem Cell Reports, 9(3), 985-998. doi:10.1016/j.stemcr.2017.06.020

Co-Author

Yue, X., Yin, J., Wang, X., Heidecke, H., Hackel, A. M., Dong, X., . . . Petersen, F. (2022). Induced antibodies directed to the angiotensin receptor type 1 provoke skin and lung inflammation, dermal fibrosis and act species overarching. Ann Rheum Dis. doi:10.1136/annrheumdis-2021-222088

Cabral-Marques, O., Halpert, G., Schimke, L. F., Ostrinski, Y., Vojdani, A., Baiocchi, G. C., … Hackel, A. ... Shoenfeld, Y. (2022). Autoantibodies targeting GPCRs and RAS-related molecules associate with COVID-19 severity. Nat Commun, 13(1), 1220. doi:10.1038/s41467-022-28905-5

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Feddahi, N., Herten, M., Tassemeier, T., Rekasi, H., Hackel, A., Haversath, M., & Jäger, M. (2021). Does Needle Design Affect the Regenerative Potential of Bone Marrow Aspirate? An In Vitro Study. Life (Basel), 11(8). doi:10.3390/life11080748

Henze, K., Herten, M., Haversath, M., Busch, A., Brandau, S., Hackel, A., . . . Jager, M. (2019). Surgical vacuum filter-derived stromal cells are superior in proliferation to human bone marrow aspirate. Stem Cell Res Ther, 10(1), 338. doi:10.1186/s13287-019-1461-0

Conference Contributions

Oral Presentations

A. Hackel. *Coping with Inflammation and Tissue Repair: A new dynamic model in natural killer cell and Mesenchymal Stromal Cell Interaction*. 47th Annual Meeting of the German Society for Immunology (DGFI), Erlangen, Germany, 2017

A. Hackel. *Optimizing the Immunoregulatory Properties of Human Mesenchymal Stromal Cells and their Extracellular Vesicles by Immunological Priming*. Graduate School of Biomedical Science (BIOME) Annual Retreat. Cologne, Germany, 2018

Poster presentation

A. Hackel. *Optimizing the immunoregulatory properties of mesenchymal stromal cells by immunological priming.* Graduate School of Biomedical Science (BIOME) Annual Retreat. Xanten, Germany, 2017

A. Hackel. Optimizing the immunoregulatory properties of mesenchymal stromal cells by immunological priming. 16th Research Day of the Medical Faculty of the University of Duisburg-Essen, Essen, 2017

A. Hackel. *Optimizing the immunoregulatory properties of mesenchymal stromal cells by immunological priming*. Keystone Symposium on Exosomes/Microvesicles. Breckenridge, Colorado, USA, 2018

A. Hackel. *Optimizing the Immunoregulatory Properties of Human Mesenchymal Stromal Cells and their Extracellular Vesicles by Immunological Priming*. 17th Research Day of the Medical Faculty of the University of Duisburg-Essen, Essen, 2018

Declaration regarding the form of publication of the thesis

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Name of doctoral candidate Signature of doctoral candidate

Date, _____ Prof. Dr. Sven Brandau _____

Name of supervisor Signature of supervisor

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Kumulative Dissertation von Herrn (cumulative thesis of Mr) Alexander M. Hackel

Autorenbeiträge (author contributions)

Titel der Publikation 1/2: **TNF-alpha and IL-1beta sensitize MSC for IFN-gamma signalling and enhance neutrophil recruitment**

Authors: **Alexander Hackel**, Aleksandra Aksamit, Kirsten Bruderek, Stephan Lang and Sven Brandau

Anteile (contributions):

- Konzept (conception) – 40 %: Beschreibung (secification) provided additional input to concept development by Sven Brandau)
- Durchführung der Experimente (experimental work) – 85 %: Beschreibung (specification). Experimantal work was conducted by the first author and by Aleksandra Aksamit.
- Datenanalyse (data analysis) – 80 %: Beschreibung (specification) Analysis was performed after discussion with Sven Brandau and Kirsten Bruderek
- Statistische Analyse (statistical analysis) – 90 %: Beschreibung (specification) Statistics were conducted by first author.
- Manuskripterstellung (writing the manuscript) – 60 %: Beschreibung (specification) First draft and follow-up drafts were prepared under continuous guidance and review by senior author Sven Brandau
- Überarbeitung des Manuskripts (revising the manuscript) – 50 %: Beschreibung (specification) Revision strategy, point-by-point and revised version of manuscript were shared between first author and senior author. Revision Experiments were shared between Alexander Hackel and Kirsten Bruderek.

Titel der Publikation 2/2**: Immunological Priming of Mesenchymal Stromal / Stem Cells (MSCs) and their Extracellular Vesicles Augments their Therapeutic Benefits in Experimental Graft-versus-Host Disease via Engagement of PD-1 Ligands**

Authors: **Alexander Hackel**, Sebastian Vollmer, Kirsten Bruderek, Stephan Lang and Sven Brandau

Anteile (contributions):

- Konzept (conception) – 40 %: Beschreibung (specification) Concept was developed together with senior author Sven Brandau, Bernd Giebel and Carsten Kirschning
- Durchführung der Experimente (experimental work) – 65 %: Beschreibung (specification). Experimantal work was conducted by the first author together with Sebastian Vollmer, Thomas Scholtysik and Rabea Madel.
- Datenanalyse (data analysis) – 90 %: Beschreibung (specification) Analysis was generated in discussion with senior author and Kirsten Bruderek
- Statistische Analyse (statistical analysis) – 90 %: Beschreibung (specification) Statistics were conducted by first author and Kirsten Bruderek.
- Manuskripterstellung (writing the manuscript) – 75 %: Beschreibung (specification) Was written by first author in close mentorship by senior author.
- Überarbeitung des Manuskripts (revising the manuscript) – 70 %: Beschreibung (specification) Revision strategy, point-by-point and revised version of manuscript were shared between first author and senior author.

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Unterschrift Betreuer Unterschrift Doktorand

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