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Inducible paracrine factors from peripheral blood mononuclear cells modulate immunological function

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Declaration

I, Dragan Copic, hereby declare that the present thesis “Inducible paracrine factors from peripheral blood mononuclear cells modulate immunological functions” is a result of my own scientific work and has not been submitted for any other degree or academic qualification.

The use of AI tools with large language model characteristics was solely restricted to DeepL. Usage was restricted to paraphrasing of single words and did not involve any form of AI assisted writing in the sense of generating passages of text.

This dissertation was completed at the Department of Thoracic Surgery as part of the PhD program in "vascular biology" in additional collaboration with the Department of Dermatology at the Medical University of Vienna. The underlying work was supervised by Univ. Prof. Dr. med. univ. Hendrik Jan Ankersmit, MBA and co-supervised by Assoc. Prof. Priv.-Doz. Dr. Michael Mildner, Phd. The projects were financed by the Aposcience AG and peer reviewed third party funding as disclosed in the funding sections of the respective manuscripts.

Two separate publications accepted in the journals Pharmaceutics and Cells, respectively, serve as the basis of this thesis. Given the stringent formatting requirements and file size limitations imposed by the Medical University of Vienna only the main manuscripts of the respective publications were included in this thesis. All supplementary information is available in the online version of the publications arising from this thesis. The published work is licensed under the Creative Commons Attribution 4.0 International License allowing reprint of the work within this thesis.

The publications that serve as the foundation of this thesis include the lists of authors and indicate their explicit contribution to the studies.

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DeepL

Abstract English

The cell-free secretome of γ-irradiated peripheral blood mononuclear cells (PBMCsec) and antithymocyte globulin (ATG) display diverse tissue regenerative and immunomodulatory effects. Using single-cell RNA sequencing (ScRNAseq) we identify the induction of secondary paracrine responses, which amplify and diversify their therapeutic effects. PBMCsec significantly altered monocyte transcriptional profiles, inducing 322 differentially expressed genes, including pro-angiogenic factors such as VEGFA, CXCL1, CXCL5 and the serine protease inhibitor SERPINB2. Functional assays showed that PBMCsec-stimulated plasma inhibits urokinase activity and protects endothelial barrier integrity. This identifies PBMCsec not only as a mixture of regenerative molecules, but also an initiator of host-driven regenerative cascades. For ATG, we identified the modulation of immune checkpoint pathways as an additional mode of immunosuppression expanding on its known T cell depleting properties. ATG-treated whole blood released interferon-γ (IFN-γ), which subsequently induced programmed death ligand-1 (PD-L1) on monocytes via JAK/STAT signaling. PD-L1+ monocytes inhibited CD8+ T cell proliferation and cytotoxicity *in vitro*. These results uncover an unrecognized mechanism for ATG by which it indirectly enforces peripheral tolerance.

Together, these findings support the concept that both PBMCsec and ATG function in part by reprogramming host immune cells to generate a secondary wave of regulatory or regenerative mediators.

Abstract German

Das zellfreie Sekretom γ-bestrahlter peripherer mononukleärer Blutzellen (PBMCsec) und Antithymozytenglobulin (ATG) vermitteln schützende Effekte während Gewebsschädigungen und Transplantation. In dieser Arbeit wurden mittels Einzelzell-RNA-Sequenzierung (scRNAseq) bislang unbekannte sekundäre parakrine Wirkmechanismen identifiziert, welche die therapeutischen Effekte beider Substanzen erweitern.
Exposition von humanem Vollblut mit PBMCsec führte zu einer starken transkriptionellen Aktivierung in Monozyten. Unter den 322 hochregulierten Genen fanden sich proangiogene Faktoren wie VEGFA, CXCL1 und CXCL5 sowie der Serinprotease-Inhibitor SERPINB2. Funktionelle Testungen zeigten, dass PBMCsec-stimuliertes Plasma die Aktivität von Urokinase signifikant hemmt und die Integrität der endothelialen Barriere schützt. Anhand dieser Ergebnisse lässt sich schlussfolgern, dass PBMCsec neben der Bereitstellung regenerativer Faktoren, zusätzlich eine sekundäre, durch Immunzellen vermittelte regenerative Kaskade in Gang setzt, welche zusätzliche zytoprotektive Mechanismen in Gang setzt.
Ähndlich zeigten wir hier bisher unbekannte immunosuppressive Wirkmechansimen für ATG auf. Konkret wurden diese über die Induktion immunomodulatorischer Immuncheckpoint-Signale vermittelt. Im Vollblut fanden sich nach Stimulation mit ATG erhöhte Konzentrationen von Interferon-γ (IFN-γ), welches über die JAK/STAT-Signaltransduktion die Expression von Programmed Death Ligand-1 (PD-L1) auf Monozyten induzierte. Diese PD-L1$^+$-Monozyten inhibierten *in vitro* die Proliferation und Freisetzung von Granzym B von aktivierten CD8$^+$ T-Zellen.
Zusammenfassend zeigen die Ergebnisse beider Ansätze, dass PBMCsec und ATG ihre Effekte über die Modulation körpereigener Immunzellen vermitteln. Die Induktion einer nachgeschaltenen Welle immunoregulatorischer sowie regenerativer Mediatoren stellt einen gemeinsamen, bislang unbekannten Wirkmechanismus dar, der das therapeutische Potenzial beider Substanzen bedeutend erweitert.

Publications arising from this thesis

Paracrine Factors of Stressed Peripheral Blood Mononuclear Cells Activate Proangiogenic and Anti-Proteolytic Processes in Whole Blood Cells and Protect the Endothelial Barrier

Dragan Copic, Martin Direder, Klaudia Schossleitner, Maria Laggner, Katharina Klas, Daniel Bormann, Hendrik Jan Ankersmit and Michael Mildner

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Antithymocyte Globulin Inhibits CD8+ T Cell Effector Functions via the Paracrine Induction of PDL-1 on Monocytes

Dragan Copic, Martin Direder, Katharina Klas, Daniel Bormann, Maria Laggner, Hendrik Jan Ankersmit and Michael Mildner

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Abbreviations

AEs	Adverse events
AICD	Activation-Induced Cell Death
AIRE	Autoimmune regulator
AKT	AKT serine/threonine kinase
APC	Antigen-Presenting Cell
ASMA	Alpha-Smooth Muscle Actin
BPI	Bacterial Permeability-Increasing Protein
CD	Cluster of Differentiation
CDCC	Complement-Dependent Cell Cytotoxicity
CREB	cAMP Response Element-Binding Protein
CTLA-4	Cytotoxic T-Lymphocyte-Associated Protein 4
CXCL	C-X-C motif chemokine ligand
DAMPs	Damage-associated molecular patterns
DC	Dendritic Cell
DFU	Diabetic foot ulcer
ENA-78	Epithelial-Derived Neutrophil-Activating Peptide 78
ERK 1/2	Extracellular-signal Regulated Kinases 1/2
ESRD	End-stage renal disease
FAS	First Apoptosis Signal
FLT3L	FMS-like Tyrosine Kinase 3 Ligand
FOXP3	Forkhead-Box-Protein P3
G-CSF	Granulocyte-Colony Stimulating Factor
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
GMP	Good Manufacturing Practice
Gro- α	Growth-regulated oncogene-α
HIGM	Hyper-Immunoglobulin M Syndrome
HLA	Human Leukocyte Antigen
HSC	Hematopoietic Stem Cell
ICAM	Intercellular adhesion molecule
ICOS	Inducible T Cell Co-Stimulator
IDO	Indoleamine 2,3-dioxygenase
IFN-γ	Interferon Gamma
IL	Interleukin
ITIM	Immunoreceptor Tyrosine-based Inhibitory Motif
ITSM	Immunoreceptor Tyrosine-based Switch Motif
JAK	Janus Kinase

LFA-1	Lymphocyte function–associated antigen 1
LGP2	Laboratory of Genetics and Physiology 2
LVAD	Left ventricular assist device
MAPK	Mitogen-Activated Protein Kinase
M-CSF	Macrophage Colony-Stimulating Factor
MDA5	Melanoma differentiation-associated protein 5
MHC	Major Histocompatibility Complex
MIF	Macrophage migration inhibitory factor
MINCLE	Macrophage-Inducible C-type Lectin
MoDC	Monocyte-Derived Dendritic Cell
mTEC	Medullary Thymic Epithelial Cell
MVO	Microvascular Obstruction
NETs	Neutrophil Extracellular Traps
NF-κB	Nuclear Factor Kappa B
NFAT	Nuclear Factor of Activated T-cells
NLR	NOD-like Receptor
NOD	Nucleotide-binding Oligomerization Domain
NSAIDs	Non-Steroidal Anti-Inflammatory Drugs
OGD	Oxygen-glucose deprivation
PAD4	Peptidyl Arginine Deiminase 4
PAMPs	Pathogen-associated molecular patterns
PBMC	Peripheral Blood Mononuclear Cell
PBMCsec	Secretome of γ-Irradiated Peripheral Blood Mononuclear Cells
PD-1	Programmed Cell Death Protein 1
PD-L1	Programmed Cell Death Ligand 1
PI3K	Phosphoinositide 3-Kinase
PRR	Pattern Recognition Receptor
RLR	RIG-I-like Receptor
ROS	Reactive Oxygen Species
SCF	Stem Cell Factor
SHP-2	SCR-Homology Region 2 Domain-Containing Phosphatase 2
TAP	Transporter Associated with Antigen Processing
TCR	T Cell Receptor
TFH	T Follicular Helper
TGF	Transforming Growth Factor
TGF-β	Transforming Growth Factor Beta
TIMI	Thrombolysis in Myocardial Infarction

TLR	Toll-Like Receptor
TNFR	Tumor Necrosis Factor Receptor
TPO	Thrombopoietin
T_{reg}	Regulatory T cell
VASP	Vasodilator-Stimulated Phosphoprotein
VEGF	Vascular Endothelial Growth Factor
VLA-4	Very late antigen-4
ZAP70	Zeta-Chain-Associated Protein Kinase 70

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CHAPTER ONE: INTRODUCTION

1. The Immune system – An overview of its organization, development, and cellular components

The immune system is an intricate network of organs, tissues, and cells (Parkin & Cohen, 2001). Primary lymphoid organs – e.g. bone marrow and thymus – are distinguished from secondary lymphatic tissues such as the spleen, lymph nodes, tonsils and the aggregations of lymphoid tissues located in the gastrointestinal and respiratory tracts (Grossi, 1988). Their respective roles in production, differentiation and maturation of immune cells are clearly defined and underlie a coordinated and tightly regulated order (Grossi, 1988; Parkin & Cohen, 2001). Generally, the immune system can be branched into innate and adaptive immunity. Regarding the cellular components, innate immunity comprises mainly cells of myeloid lineage (e.g. leukocytes, monocytes/macrophages, dendritic cells amongst others), while lymphoid cell types (T lymphocytes and B lymphocytes) constitute adaptive immunity (Chaplin, 2010; Cooper & Alder, 2006).

The functional unit at the origin of all myeloid and lymphoid cell types are hematopoietic stem cells (HSCs) (Chaplin, 2010). HSCs reside within the bone marrow niche and represent a specific fraction of cells characterized by their ability for self-renewal and multipotency (Yu & Scadden, 2016). These features are essential to ensure lifelong persistence and enable differentiation into different cell types of peripheral blood. Development and differentiation from HSCs into myeloid and lymphoid progenitor cells and subsequently terminally committed cell types is hierarchical and requires a complex interplay of cell-to-cell interactions as well as soluble growth factors present in the bone marrow niche (Pinho & Frenette, 2019; Yu & Scadden, 2016). Stromal cells, endothelial cells and osteoblasts are important cell types involved in the release of paracrine factors that streamline differentiation of HSCs into committed cell lineages. Stem cell factor (SCF, kit ligand) is a key factor involved in this process as it promotes survival, maintenance and proliferation of HSCs (Pinho & Frenette, 2019). Together with thrombopoietin (TPO) and FLT3 ligand (FLT3L) this leads to expansion of early myeloid and lymphoid progenitors (Chen *et al*, 2004; Morrison & Scadden, 2014). At this point, more lineage specific factors drive the commitment and terminal differentiation of these cells. As such, Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) is required for granulopoiesis, macrophage and dendritic cell formation. Granulocyte-Colony Stimulating Factor (G-CSF) stimulates generation of neutrophil granulocytes while Macrophage Colony-Stimulating Factor (M-CSF) is specifically involved in macrophage differentiation and maturation. In addition, different interleukins (e.g. IL-3, IL-6, IL-7) play crucial roles in myeloid and lymphoid cell development (Tarr, 1996).

Once myeloid and lymphoid precursor cells conclude their differentiation towards more distinct cell types these cells are released into systemic circulation. At this point cells of the innate immune system (leukocytes, monocytes, natural killer cells and dendritic cells amongst others) possess a repertoire of non-specific mechanisms to combat pathogens (Kaur & Secord, 2021), while lymphocytes (T-lymphocytes, B-lymphocytes) must undergo a more target adaptive maturation to establish efficient and lasting immunity (Bonilla & Oettgen, 2010).

2. Innate immunity in host defense

Maintenance of host defense by recognition and elimination of exogenous pathogens, while maintaining self-tolerance to prevent autoimmune reactions are central functions of the immune system (Kaur & Secord, 2021). Therefore, immunological responses underlie a strict coordination of various checkpoints involving cellular and humoral units and make use of a plethora of mechanisms to effectively and lastingly protect against a wide range of infectious triggers while avoiding autoimmune mediated tissue damage (Medzhitov & Janeway, 1997). The immune systems response to exogenous stressors – e.g. bacterial or viral infections – is multi-layered (Medzhitov & Janeway, 1997). The first line of defense is set by the innate immune system and allows for rapid detection and non-specific responses to pathogens. This prompt response is crucial due to the brief generation times of many bacterial and viral agents, allowing the innate immune system to maintain host defense while facilitating the development of a phased and target-specific adaptive immune response (Divangahi *et al*, 2021).

2.1 Mechanisms of pathogen recognition and immune responses in innate immune cells

Neutrophil granulocytes, monocytes/macrophages and natural killer cells recognize common features of invaders and initiate an immediate attack by means of targeted phagocytosis or release of cytolytic enzymes (Reeves *et al*, 2002; Thomas *et al*, 2000). A set of evolutionary highly conserved proteins – so called pattern recognition receptors (PRRs) – serve in host innate immunity for distinction of self-tissue and recognition of microbe specific pathogen-associated molecular patterns (PAMPs) (Medzhitov, 2013; Takeuchi & Akira, 2010). Generally, two groups of PRRs are distinguishable (Takeuchi & Akira, 2010). They involve secreted or circulating proteins/peptides in addition to cell-associated detectors that trigger the transduction of an intracellular signal upon recognition of PAMPs (Medzhitov, 2013; Takeuchi & Akira, 2010).
Prominent circulating PRRs are the complement factor C1q (Bohlson *et al*, 2007), pentraxins

(Mantovani *et al*, 2003), collectins (Sastry & Ezekowitz, 1993), lectins and antimicrobial peptides (Takeuchi & Akira, 2010), which in turn can be subdivide into defensins, cathelicidins and bacterial permeability-increasing proteins (BPI) (Cederlund *et al*, 2011). These circulating PRRs bind to pathogenic microbes to either directly mediate microbial killing, act as tags to facilitate pathogen detection (opsonization) via effector innate immune cells or serve as cross-linkage to transmembrane proteins leading to immune cell activation (Tietze *et al*, 2006).

Cell-associated PRRs present at cell membrane, cytoplasmic or lysosomal levels and include transmembrane receptors such as C-type lectin receptors (dectin-1 and dectin-2) (Linehan *et al*, 2000b), macrophage scavenger receptor and macrophage mannose receptor (Linehan *et al*, 2000a), Nucleotide-binding oligomerization domain (NOD) like receptors (NLRs, NOD1 and NOD2) (Girardin *et al*, 2003; Inohara *et al*, 2001), macrophage-inducible C-type lectin (MINCLE) (Richardson & Williams, 2014), RIG-1-like receptors (RLRs) (Takeuchi & Akira, 2008), melanoma-associated differentiation protein 5 (MDA5) (Takeuchi & Akira, 2008) and laboratory of genetics and physiology 2 (LGP2) (Reikine *et al*, 2014). Another group of highly conserved PRRs are the different members of the toll-like receptor family (TLRs) (Schnare *et al*, 2006). TLRs function either as transmembrane (TLRs 1, 2, 4, 5, 6) or intracellular receptors (TLRs 3, 7, 8, 9, 10) and are highly specific in detection of PAMPs (Biondo *et al*, 2012; Schnare *et al.*, 2006). Expression of PRRs is constitutive and can be modulated in response to activation to amplify cellular responses (Lee *et al*, 2019).

Ligation of a PRR by a PAMP triggers transcription of pro-inflammatory genes and the subsequent release of paracrine mediators (Park *et al*, 2004).

Prolonged exposure to inflammation consequently leads to cell death and tissue necrosis during which additional intracellular mediators (i.e. nuclear contents, mitochondria etc.) are released into the surroundings (Vringer & Tait, 2023; Wickman *et al*, 2013). These serve as danger signals for surrounding cells and tissues also known as damage-associated molecular patterns (DAMPs) (Ma *et al*, 2024). DAMPs are detected by PRRs on innate immune cells to augment the inflammatory response. Their activation leads to recruitment of additional immune cells to the inflamed site to aid in its resolution by means of phagocytosis or release of inflammatory and cytoprotective paracrine factors (Ma *et al.*, 2024).

2.2 Innate immune cells and their effector functions

Neutrophil granulocytes represent the most abundant immune cell population in human peripheral blood and are typically amongst the first cells recruited to a site of inflammation or bacterial/fungal infection (Rehaume & Hancock, 2008). This is facilitated by different types of chemotactically active substances including different chemokines, factors related to the complement system, bacterial oligopeptides and fatty-acid derivatives amongst others

(Harvath, 1991). Once neutrophils reach their target site, they execute their effector functions (Mantovani *et al*, 2011). Neutrophils are professional phagocytes capable of ingestion and lysosomal digestion of captured pathogens (Kerrigan *et al*, 2009). Additionally, activated neutrophils release intracellular granules filled with antimicrobial peptides, proteases and other factors (Borregaard *et al*, 1993). The contents of these granules are highly toxic to bacterial and fungal pathogens, as they disrupt cell membranes through degradation and chelate iron, thereby depriving microbes of this essential element and hindering their growth (Cramer *et al*, 1985). Another defense mechanism employed by neutrophils is the release of neutrophil extracellular traps (NETs) (Yousefi *et al*, 2009). They are net-like structures released by neutrophils following stimulation with bacterial antigens or inflammatory cytokines (Fuchs *et al*, 2007). They contain DNA, citrullinated histones and granule proteins like elastase and myeloperoxidase (Schauer *et al*, 2014). NETs immobilize pathogens to prevent them from spreading and facilitate their clearance by other immune cells (Granger *et al*, 2019).

Monocytes constitute about 5-10% of peripheral blood leukocytes (Auffray *et al*, 2009). They circulate within blood and are responsible for immune surveillance (van Furth & Cohn, 1968). They are highly adaptable with a broad spectrum of functions ranging from pathogen recognition, transmigration, initiation of inflammatory responses, phagocytosis and paracrine factor mediated actions to influence adaptive immune responses (Jakubzick *et al*, 2017; Serbina *et al*, 2008). Traditionally, three classes of monocytes can be distinguished based on surface expression of the cluster of differentiation surface proteins CD14 and CD16 as well as their respective functions (Thaler *et al*, 2016). Classical monocytes (CD14++/CD16-) are the most abundant out of the three (Tak *et al*, 2017). They act as rapid responders to infection or injury as they exhibit high phagocytic activity and produce various pro-inflammatory cytokines to further amplify the immune response by recruitment of additional immune cells (Tak *et al.*, 2017). Non-classical Monocytes (CD14+ CD16++) partake in maintenance homeostasis of the vascular endothelium (Ancuta *et al*, 2009). They patrol the luminal side of the endothelium to detect and remove damaged cells, clear apoptotic cells and debris to prevent excessive inflammation and ameliorate tissue-damage (Quintar *et al*, 2017). They impact vascular repair and maintenance of blood vessel integrity and endothelial health (Quintar *et al.*, 2017). Their role in tissue repair and resolving inflammation is exemplified by the production of anti-inflammatory cytokines (Thomas *et al*, 2015). Intermediate monocytes (CD14++ CD16+) combine features of the other classes and are associated with chronic inflammatory conditions as present in chronic infections and cardiovascular diseases (Hijdra *et al*, 2013). They are potent producers of pro-inflammatory cytokines and bridge innate to adaptive immunity as they have antigen presenting properties

that result in activation of T cells. (Hijdra *et al.*, 2013) Monocyte functions are not exclusively limited to defense against exogenous pathogens as they are also crucially involved in tissue-repair after injury, immune modulation and maintenance of vascular health (Duffield *et al*, 2013). Following the recognition of pathogen/danger signals monocytes transmigrate from the circulation towards the affected tissues. Based on the encountered local environment and tissue-specific requirements for resolution they differentiate into specialized macrophages or monocyte-derived dendritic cells (Delneste *et al*, 2003). M-CSF promotes differentiation into macrophages, while GM-CSF favors dendritic cell formation (Becker *et al*, 1987).
Like monocytes, macrophages are highly versatile and display distinct functions based on their surroundings (Mantovani *et al*, 2004). Polarization into predominantly pro-inflammatory M1 macrophages occurs in response to inflammatory stimuli (Murray *et al*, 2014). M1 macrophages perform phagocytosis, production and release of reactive oxygen species, nitrate intermediates and pro-inflammatory cytokines to eliminate pathogens and resolve inflammation (Krausgruber *et al*, 2011). Anti-inflammatory M2 macrophages are key contributors in wound healing, tissue remodeling, and resolution of inflammation by release of IL-10 and TGF-β along with various other anti-inflammatory cytokines and growth factors (Porta *et al*, 2009).

Monocyte-derived dendritic cells (moDCs) are specialized antigen-presenting cells that capture and process exogenous and endogenous antigens to present them to lymphocytes (León *et al*, 2005). PPRs play an essential role in moDCs recognition of antigens in peripheral tissues and facilitate subsequent capture (Mellman & Steinman, 2001). Depending on structural complexity of the captured antigen, different mechanisms of antigen uptake are available. The three main mechanisms of antigen uptake are phagocytosis, macropinocytosis for soluble antigens and receptor-mediated endocytosis (de Baey & Lanzavecchia, 2000; Inaba *et al*, 1998; Mahnke *et al*, 2000). Furthermore, moDCs receive activating signals through PRRs that trigger maturation and upregulation of co-stimulatory molecules such as CD80, CD86, and CD40 which help streamline T cell interaction (Cella *et al*, 1997). Activation also induces DC migration toward secondary lymphoid tissues, where they present processed antigens to T cells. Following antigen capture, moDCs initiate intracellular processing pathways to break down antigens into peptide sequences suitable for antigen presentation (Inaba *et al.*, 1998). MoDCs present processed peptides either through major histocompatibility complex (MHC) class I or class II pathways (Cresswell *et al*, 2005; Inaba *et al.*, 1998). Depending on the MHC molecule involved in peptide presentation different types of T cells are activated. Generally, cytotoxic CD8+ T cells are primarily engaged via MHC class I (Yewdell *et al*, 1999) while CD4+ T-helper cells are engaged via MHC class II (Wang, 2003). Processing of exogenous antigens results in activation of the MHC class II pathway

(Kyewski & Haskins, 2012). Antigens are internalized in endosomes within the moDCs where they fuse with lysosomes to form acidic endolysosomes (Turley *et al*, 2000). Different proteolytically active enzymes degrade the antigens into smaller peptide fragments (Blum *et al*, 2013). Simultaneously to degradation, MHC class II molecules are synthesized in the endoplasmic reticulum (Germain & Hendrix, 1991). At this stage the peptide binding site of the MHC class II molecule is temporarily blocked by an invariant chain to prevent premature loading (Cresswell, 1996). These MHC class II-invariant chain complexes are then transported to the endolysosome, where the acidic environment degrades the invariant chain. This leaves a fragment called class II-associated invariant chain peptide (CLIP) in the binding groove (Turley *et al.*, 2000). The non-classical MHC molecule HLA-DM removes CLIP, which frees up the MHC class II molecules peptide binding site to the processed antigenic peptides to bind to the MHC class II molecule (Weenink & Gautam, 1997). The peptide-loaded MHC class II complex is then transported to the cell surface, where it is displayed for recognition by CD4+ helper T cells (Weenink & Gautam, 1997).
Viral and tumor-derived proteins are typically recognized by MHC class I molecules (Calis *et al*, 2013). MoDCs can present such antigens through cross-presentation (Shen *et al*, 1997). During this process antigens are internalized as described for the MHC class II pathway but are subsequently transported from endosomes to the cytoplasmic space where the antigen is degraded by the proteasome to produce peptide fragments (Guermonprez *et al*, 2003). Next, the transporter associated with antigen processing (TAP) proteins facilitate the translocation of these peptides into the ER, where they encounter newly synthesized MHC class I molecules (Day *et al*, 1995). The peptides are loaded onto MHC class I molecules in a complex facilitated by chaperones such as tapasin, ensuring proper binding and stability (Chefalo *et al*, 2003). The peptide-MHC class I complex is then transported to the cell surface for presentation to CD8+ cytotoxic T cells. MoDCs emply these antigen processing pathways to bridge innate and adaptive immunity and form a well-coordinated immune response against a wide range of pathogens and cellular stressors (Banchereau & Steinman, 1998). Furthermore, antigen presentation provides signals necessary for T cell differentiation and activation, thereby promoting an organized and efficient transition from a non-specific innate response to a targeted adaptive one (Acton & Reis e Sousa, 2016; Banchereau & Steinman, 1998).

3. T cell tolerance

3.1 Mechanisms leading to central tolerance

Several regulatory mechanisms are employed to facilitate maintenance of tolerance to self-antigens while also upholding immune homeostasis (Cooper & Alder, 2006). As such, during the development of T and B cells in the thymus and bone marrow, respectively, these cells undergo a selection process called central tolerance (Nemazee, 2017; Sprent & Kishimoto, 2001). Developing T cells in the thymus are referred to as thymocytes (Cantor & Weissman, 1976). These cells express T cell receptors (TCR) on their surface (Takahama, 2006). During positive selection, which occurs in the cortical parts of the thymus, thymocytes capable of recognition of self-MHC molecules with a low to moderate affinity are selected for survival, while thymocytes incapable of self-recognition are eliminated (Takada & Takahama, 2015). As the selected cells transition towards the medullary sections of the thymus, thymocytes that display high-affinity binding of self-antigens presented by medullary thymic epithelial cells (mTECs) as well as dendritic cells are eliminated through negative selection by induction of programmed cell death (Takahama, 2006). The transcription factor Autoimmune Regulator (AIRE) (Ledbetter *et al*) is essential in this process as it enables mTECs to express a diverse array of tissue-restricted antigens to transitioning thymocytes (Anderson *et al*, 2002; Sansom *et al*, 2014). Tissue-restricted antigens cover a broad spectrum of self-antigens typically found outside of the thymus (Sansom *et al.*, 2014). These selections ensure that developing T cells possess a TCR capable of recognition of self-MHC without strongly recognizing the specific self-antigens presented (Takada *et al*, 2015). Thereby, this dual-staged selection during central tolerance is pivotal to prevent maturation of autoreactive T cells thereby reducing risk for autoimmunity (Kondo *et al*, 2017).

3.2 Mechanisms leading to peripheral tolerance

Thymocytes that fulfill the criteria of successful binding of self-peptides with low to moderate affinity to self-peptides progress to the mature T cell stage and can be released into the periphery as a mature, naive T cell ready to respond to foreign antigens (Redmond & Sherman, 2005). In instances where self-reacting T cells escape central tolerance, mechanisms of peripheral tolerance serve as an additional safeguarding mechanism to complement central tolerance (Grossman & Paul, 2001). Peripheral tolerance in T cells relies on several mechanism designed to keep self-reactive T cells in check without impairing the organism's ability to mount an adequate immune response against foreign pathogens (von Boehmer & Jaeckel, 2001). These mechanisms involve induction of functional unresponsiveness (anergy), active suppression, inhibitory co-stimulation and deletion of self-reactive T cells (Jordan *et al*, 2000).

Anergy describes a state of T cell unresponsiveness characterized by lack of a co-stimulatory signal in the presence of TCR engagement to a self-antigen (Deeths *et al*, 1999; Schwartz, 2003). In the context of peripheral tolerance presentation of self-antigens by non-professional APCs without a modulating co-stimulatory signal result in anergy (Boussiotis *et al*, 1997). However, reversal of anergic states upon prolonged antigen exposure in inflammatory environments with strong co-stimulation have been reported (Dekeyser *et al*, 2017). Therefore, peripheral tissues with high frequencies of co-stimulatory receptors – which underly a dynamic modulation – depend on different modes of T cell regulation (Lechler *et al*, 2001; Macián *et al*, 2004).

Regulatory T cells (T_{regs}) are a specialized subset of $CD4^+$ T cells and regulators of peripheral tolerance (Martin *et al*, 2003). They are active suppressors of self-reacting T cells inhibiting their functions via cytokine secretion as well as direct cell-cell interaction (Chatila, 2005). Most T_{regs} express the transcription factor FOXP3 that drives their development as well as their immunosuppressive functions (Hori *et al*, 2003). By release of immunosuppressive cytokines such as IL-10 and TGF-β T_{regs} potently inhibit T cell proliferation and reduced production of proinflammatory cytokines by their target cells (Maloy & Powrie, 2001; Marie *et al*, 2005). Their actions extend beyond release of paracrine factors, as T_{regs} repress T cell activation via CTLA-4 dependent co-inhibition as well as CD25 (IL-2-receptor), which captures IL-2 and interferes with T cell proliferation (Krummel & Allison, 1996; Perez *et al*, 1997).

Like T_{regs} other cells of the immune system modulate T cell autoreactivity by inhibiting co-signaling pathways (Fraser *et al*, 1993). The immune-checkpoint CTLA-4 competes with CD28 for binding of CD80/CD86 while PD-1 binds its ligand PD-L1 to interfere with CD28 signaling (Krummel & Allison, 1995; O'Donnell *et al*, 2017). They interfere directly with the immune synapse between T cells and APCs, thereby alleviating excessive or prolonged T cell activation allowing for localized responses without widespread immunosuppression (Freeman *et al*, 2000).

Persistent and repeated exposure to self- or foreign-antigen chronically activates T cells leading to upregulation of death-receptors which mediate Activation-Induced Cell Death (AICD) mediated by specific receptor-ligand interactions (Green *et al*, 2003). The engagement results in induction of programmed cell death to eliminate these auto- and overreacting T cells (Shi *et al*, 1991). Chronic activation of T cells results in upregulation of the death receptors FAS (CD95) and Tumor Necrosis Factor Receptor 1 (TNFR1) (Ankersmit *et al*, 1999). Ligation of these receptors by their respective ligands FAS-L (CD95-L) and TNF-α results in programmed cell death of the activated T cell mediated by the extrinsic apoptotic pathway (Dhein *et al*, 1995).

Aberrations in T cell activation secondary to increased apoptotic turnover due to AICD have been reported in cohorts of patients suffering from end-stage renal disease (ESRD) on renal replacement therapy in the form of either hemo- or peritoneal dialysis as well as conservatively managed ESRD patients (Ankersmit *et al*, 2001; Moser *et al*, 2003). Similar findings had been reported in patient with end-stage heart failure subjected to left-ventricular assist device (LVAD) therapy (Ankersmit *et al.*, 1999). When compared to heart-failure patients kept on optimal supportive medical therapy, patients receiving LVAD displayed decreased levels of circulating CD4 T cells while also revealing increased levels of circulating soluble CD95 together with higher susceptibility for CD4 and CD8 T cell apoptosis (Ankersmit *et al.*, 1999). Loss of adaptive immune cells can thus worsen the already existing susceptibility in these patient cohorts for complications like fatal infections including sepsis which serves as an independent trigger for AICD and therefore perpetuates this process further (Roth *et al*, 2003).

Observational data from a cardiac transplant cohort receiving antithymocyte globulin (ATG) induction therapy on top of calcineurin inhibitor, antiproliferative agent and glucocorticoid therapy revealed freedom of rejection free survival in 86% of the 618 cardiac transplant recipients after 1-year follow up (Ankersmit *et al*, 2002). Investigation of changes in surface expression of death-receptors in T cells in a subset of these cardiac allograft recipients revealed significantly increased levels of CD95, CD95L and soluble TNFR1 when compared to control patients with end-stage heart failure (Ankersmit *et al.*, 2002). These findings were accompanied by a heightened susceptibility of patients T cells to undergo apoptosis when stimulated with monoclonal antibodies against IL-2R, OKT3 and polyclonal ATG *ex vivo* (Ankersmit *et al.*, 2002).

4. Mechanisms of T cell activation

T cell activation is a highly regulated process that requires coordinated antigen recognition in addition to secondary signaling (Kreileder *et al*, 2021). The latter is modulated by co-stimulatory or co-inhibitory molecules (Smith-Garvin *et al*, 2009). This dual-staged signal interpretation ensures that regulation of T cell responses is in accordance with the immunological context, while unintended responses that result in autoimmunity are restricted (Garcia & Ismail, 2020). T cells express the T cell receptor (TCR), a heterodimeric protein complex, on their cell surface (Riley *et al*, 2002). The vast majority of TCRs are configured as a dimer formed by a highly variable alpha (α) and beta (β) chain (αβ T cells), while about 5% of T cells express TCRs formed by variable gamma (γ) and delta (δ) chains (γδ T cells) (Clayton *et al*, 1992). Together with invariant CD3 molecules TCRs form the TCR complex (Rojo *et al*, 2008). Binding of the TCR to either an exogenous antigenic peptide via MHC class II on the surface of APCs or endogenous peptides by MHC class I on any other cell (excluding erythrocytes) provides the first activation signal (König, 2002). However, T cell signaling initiated by TCR-MHC interaction (signal one) is insufficient to effectively activate T cells (Ledbetter *et al.*, 1990). A second activating or inhibitory signal (signal two) is required to modulate the T cell (Grakoui *et al*, 1999). Depending on the co-stimulatory signal this can result in activation, anergy (non-functionality due to missing co-stimulatory signal) or initiation of self-reactivity (autoimmunity) (Clements *et al*, 1999).

4.1 Co-stimulatory signals

Co-stimulation via signal two regulates effector functions of T cells (Schwartz, 1992). This allows for a controlled modulation of T cell activation, differentiation and proliferation leading to clonal expansion (Sears & Nevins, 2002). Co-stimulatory signals are provided by receptor-ligand interactions between the T cell and an APC (Smith-Garvin *et al.*, 2009). CD28 is one of the best studied co-stimulatory receptors. Binding to either of its ligands CD80 (B7-1) and CD86 (B7-2) - present on the surface of dendritic cells - serves as an activating co-stimulatory signal promoting T cell activation and production of interleukin-2 (IL-2), which in turn is a secreted cytokine driving T cell proliferation and survival (Orabona *et al*, 2004). Expression of CD28 is constitutively active and already present even on naïve T cells (T cell which is yet to encounter its cognate antigen) (Guo *et al*, 2008). Binding of CD28 to CD80/CD86 while TCR-MHC activation enhances TCR signaling (Acuto & Michel, 2003). This results in the activation of pro-survival pathways involving MAPK and PI3K-AKT which translate to promotion of T cell survival, proliferation and differentiation (Kane *et al*, 2001). Another essential pathway upregulated by interaction between CD28 and CD80/CD86 involves NF-κB which results in enhanced cytokine production (Coudronniere *et al*, 2000).

Another activating co-stimulatory receptor is the Inducible T Cell Co-Stimulator (ICOS) (Richter & Burdach, 2004). As suggested by its name, ICOS is an inducible factor not constitutively expressed on T cells. More specifically, induction of ICOS is mediated upon activation of antigen-experienced CD4+ effector T cells (Ozkaynak *et al*, 2001). Receptor-Ligand interaction of ICOS and ICOS-L - present on myeloid and plasmacytoid DCs, macrophages, B cells as well as on non-hematopoietic tissues - is required to stimulate differentiation of CD4+ helper cells into T follicular helper cells (T_{FH}) (Panneton *et al*, 2023). Highest frequencies of ICOS-expressing T cells are detected within the germinal centers of secondary lymphatic organs (Nurieva, 2005). This specialized subset of CD4+ T cells regulate humoral responses by B cell activation and impact stimulation of antibody production (Xu *et al*, 2013). Downstream signaling involves activation of PI3K-AKT for promotion of early T cell activation, T_{FH} cell differentiation and migration to the follicle and functional maintenance of germinal centers (Okamoto *et al*, 2004).

CD40 is a member of the TNFR superfamily and expressed on various cell types including, macrophages, DCs, B cells, platelets, activated epithelial as well as endothelial cells (van Kooten & Banchereau, 2000). Upon binding of CD40 ligand (CD40L, CD154), which is expressed on activated CD4+ T cells, macrophages, mast cells, thrombocytes, and vascular endothelial cells, the CD40/CD40L interaction sets off a signaling cascade leading to activation of transcription factors involved in cell survival, activation and differentiation (Elgueta *et al*, 2009).
Ligation of CD40/CD40L on macrophages and DCs results in upregulation of surface activation molecules (e.g. CD69, CD80 and CD86) to enhance their regulatory functions and induce cytokine secretion (Grewal & Flavell, 1996). Together these mechanisms impact the regulation of CD4+ T cell functions and CD8+ T cell cross-priming and activation (Hoffmann *et al*, 2001). Furthermore, CD40:CD40L activation is essential in B cell activation by regulation of germinal center formation, antibody production, and the generation of long-term immunity (Kehry, 1996). Given these effects, clinical trials in patients with systemic lupus erythematosus were designed to evaluate the influence of a humanized monoclonal anti-CD40L antibody (hu5c8) on disease course (Boumpas *et al*, 2003). However, these had to be terminated prematurely due to a safety signal for increased rate of thromboembolic events reported in the intervention group that were mechanistically attributed to platelet activation and aggregation from Fc-mediated cross-linking (Robles-Carrillo *et al*, 2010).

A deeper understanding of the involvement of CD40/CD40L axis in T cell dependent B cell responses was elucidated by studies in patients affected by hyper-IgM syndrome (HIGM) (Callard *et al*, 1993). In this X-linked immunodeficiency syndrome patients B cells with loss-of-function mutations to either of the receptor-ligand pair are incapable of immunoglobulin class switching or affinity maturation, therefore limiting their ability to combat opportunistic pathogens resulting in increased susceptibility to infections (DiSanto *et al*, 1993). Interestingly active forms of CD40 as well as CD40L have also been detected in circulation suggesting para- and autocrine mechanisms in addition to the cell-cell dependent forms (Choi *et al*, 2024).

Moreover, a typical feature of co-stimulatory signals is the upregulation of additional regulators to streamline the immunological response further. CD40/CD40L dependent upregulation of OX40 (CD134) is a classic example of this signal amplification (Ohshima *et al*, 1997). OX40 has been detected in various T cell subsets where activation via OX40 ligand (OX40L) ensued downstream pathway activation of PI3K-AKT, MAPK, NF-κB and NFAT (Redmond *et al*, 2009). The activation of these pro-survival pathways translates to enhanced proliferation and differentiation of CD4+ T cell subsets – including regulatory T cells (T_{regs}) - and augmented cytokine production (Redmond *et al.*, 2009).

The co-stimulatory receptor 4-1BB (CD137) is highly expressed on CD8+ T cells upon activation via 4-1BBL (Kim *et al*, 2003). Its activation drives cytotoxic T cell responses resulting survival and proliferation of activated T cells (Kim *et al.*, 2003). Therefore, this pathway ensues sustained cytotoxic CD8+ T cell responses crucial for resolution of viral infections and tumor immunity (Ye *et al*, 2014).

4.2 Co-Inhibitory Signals and Immune Regulation

Co-inhibitory receptors, or immune checkpoints, are essential to restrain T cell activation and maintain self-tolerance (Murakami & Riella, 2014). These receptors are upregulated on activated T cells to limit immune responses, prevent excessive inflammation and reduce the risk of autoimmunity (Fife & Bluestone, 2008).

Cytotoxic T-Lymphocyte-Associated Protein 4 (CTLA-4) is a key co-inhibitory receptor upregulated on activated T cells and constitutively expressed in T_{regs} (Walunas *et al*, 1994). It is the functional counterpart to CD28 and competes for binding to the shared ligands CD80 and CD86 on APCs (Walunas *et al*, 1996). Due to its higher binding affinity for CD80 and CD86 it efficiently outcompetes CD28 resulting in a dampened T cell response. More specifically, binding of CTLA-4 to either ligand has been shown to result in degradation and trans-endocytosis of the ligands expressed on APCs making them unavailable for CD28 engagement (Kennedy *et al*, 2022; Qureshi *et al*, 2011). The relative distribution pattern of CTLA-4 – 10% are expressed on the cell membrane as opposed to the remaining fraction

present in endocytic space – supports this proposed mode of action (Qureshi *et al*, 2012). Signaling via CTLA-4 results in inhibition of T cell effector functions such as production of IL-2 and cell-cycle progression (Oosterwegel *et al*, 1999). Thereby, CTLA-4 dependent co-inhibition effectively slows T cell activation and proliferation (Vandenborre *et al*, 1999). In vivo animal studies of CTLA-4 deficient mice revealed strong dysregulation of lymphocytic autoreactivity resulting in diffuse tissue inflammation (Chambers *et al*, 1997). Furthermore, CTLA-4 has been identified as a crucial component of T_{regs} as it is constitutively expressed on these cells and mediates suppression of other T cell subsets to maintain immune homeostasis (Schmidt *et al*, 2012).

Programmed Cell Death Protein 1 (PD-1) is another immune checkpoint that regulates T cell function to prevent excess and overt activation (Riella *et al*, 2012). It is a member of the CD28 receptor family and binds to its ligands Programmed Cell Death Ligand 1 (PD-L1) and Programmed Cell Death Ligand 2 (PD-L2) (Carter *et al*, 2002; Latchman *et al*, 2001). While CTLA-4 primarily acts in lymphoid tissues during the initial stages of T cell activation PD-1 functions primarily at effector sites limiting T cell activation and effector function during chronic immune responses or when T cells encounter persistent antigen stimulation (Parry *et al*, 2005). PD-1 is expressed on the cell surface of various cell types including T cells, B cells and myeloid cells, while the natural ligands are present on APCs, epithelial and endothelial cells and tumor cells amongst others (Keir *et al*, 2008). Furthermore, functionally active soluble forms of either PD1 or PD-L1 – resulting from splice variants or cell surface shedding – have been reported (Niu *et al*, 2022). Expression of PD-1 and PD-L1/PD-L2 is regulated in response to activating stimuli (Ishida *et al*, 1992). Prolonged antigen exposure and TCR modulation as encountered in chronic infections or tumor microenvironments are potent drivers of T cell activation and therefore modulate expression of PD-1 in affected T cells (Lin *et al*, 2024).
Ligation of PD-1 by either membrane bound or soluble active forms of PD-L1/PD-L2 during simultaneous TCR complex engagement influences T cell responses via interference with CD28-mediated as wells as TCR complex-mediated signaling (Hui *et al*, 2017; Mizuno *et al*, 2019). Following phosphorylation of the cytoplasmatic immunoreceptor tyrosine-based inhibitory motif (ITIM) and the immunoreceptor tyrosine-based switch motif (ITSM) domains Src Homology Region 2 Domain-Containing Phosphatase 2 (SHP-2) is recruited to the ITSM to negatively regulate key molecules downstream of CD28 and the TCR (Chemnitz *et al*, 2004; Yokosuka *et al*, 2012). Amongst those Zeta-chain-associated protein kinase 70 (ZAP70), Linker of Activation in T cells and the TCR subunit CD3ζ are amongst the most thoroughly studied (Sheppard *et al*, 2004). Additionally, PD-1 signaling interferes with PI3K-AKT, mTOR and Ras/MAPK (Patsoukis *et al*, 2012). These are regulators of T cell

metabolism, cytokine production, proliferation, cytotoxicity and survival inhibited by PD-1 activation (Mizuno *et al.*, 2019).

The PD-1/PD-L1 pathway underlies a hierarchical order, where CD28 signaling is suppressed more potently relative to TCR signaling (Arasanz *et al*, 2017). This selective inhibition of the co-stimulatory signal mediated by CD28 at the immune synapse is a critical feature of PD-1 function with particular importance regarding T cell exhaustion (Chen & Flies, 2013). States of prolonged CD28-mediated T cell activation result in overreaction of the immune system (Saeidi *et al*, 2018). In cases of chronic antigen exposure, as encountered in persistent viral infections or within tumor microenvironments, PD-1 expression is sustained on T cells (Kahan *et al*, 2015). Activation of PD-1 consequently leads to a state known as T cell exhaustion (Wherry & Kurachi, 2015). Exhausted T cells exhibit reduced effector functions, including decreased production of IL-2, IFN-γ, and TNF-α, reduced proliferative capacity as well as diminished cytotoxic capabilities (Yi *et al*, 2010). T cell exhaustion aids in maintenance of immune tolerance and limits tissue damage during chronic immune responses (Blackburn *et al*, 2010). However, it also compromises the immune system's ability to mount an effective immune response against chronic infections or tumors (Wherry, 2011).

5. Immunomodulation – General overview

Immunomodulation involves processes that influence the activity or responsiveness of the immune system (Sangeetha Vijayan *et al*, 2024). This modulation may lead to an enhanced or suppressed immune response (Vesely *et al*, 2011). It is essential in maintaining health and resolving disease states. Immune regulation is governed by a tightly coordinated network of tolerance mechanisms, feedback inhibition, and immunoregulatory cells (Sangeetha Vijayan *et al.*, 2024). These mechanisms unite to prevent overactivation of the immune system with collateral tissue damage and uphold host defense to infectious pathogens (Chaplin, 2010). However, dysregulation of these immunological safeguards contributes to the pathogenesis of a broad spectrum of diseases (Bonilla & Oettgen, 2010; Kaur & Secord, 2021). Chronic amplification of immune responses predisposes for inflammatory and autoimmune conditions. In contrast to that, excessive suppression, as seen in immunodeficiency, compromises pathogen defense and tumor surveillance (Ray & Yung, 2018). Therefore, a central dogma of effective immunomodulation is reverse inadequate immunological responses by either dampening pathogenic inflammation or enhancement of insufficient immune responses (Ray & Yung, 2018).

Therapeutic immunomodulation is a key element of modern clinical practice and allows for a distinction between three major categories of interventions available for use.

Small-molecule agents such as corticosteroids, calcineurin inhibitors, non-steroidal anti-inflammatory drugs (NSAIDs), and kinase inhibitors, amongst various others, with inflammatory pathways by broad spectrum of mechanisms to suppress them (Cain & Cidlowski, 2017; Lee *et al*, 2010; Matsuda & Koyasu, 2000). Thus, they are well-established treatment options and remain widely used across autoimmune, allergic, and transplant-related indications (Strehl *et al*, 2019).

Biologic agents offer a more target specific approach to immunomodulation (Lee *et al.*, 2010). This category of immunomodulators includes monoclonal and polyclonal antibodies as well as recombinant cytokine inhibitors (Lee *et al.*, 2010). These enable precise targeting of specific immune pathways that can be exploited for interference with pro-inflammatory states – e.g. anti-TNF-α and anti-IL-6 antibodies – or to enhance immune activation through checkpoint inhibition in oncology (Lee *et al.*, 2010; Mitoma *et al*, 2018).

Cell-based therapies involve the third major category of immunomodulatory interventions (Song *et al*, 2020). They represent a more dynamic approach by introducing either living cells or paracrine factors released upon activation that confer modulating actions on immune cells in a context-dependent manner. The spectrum of cell types exploited for cell-based therapies offers a broad range. Use of mesenchymal stromal cells (MSCs) for tissue regeneration and immunomodulation is an extensively studied field (Galipeau & Sensébé, 2018). These cells are of particular interest given their plasticity and pluripotency (Dominici *et*

al, 2006). However accumulating evidence from preclinical trials suggests that their immunomodulatory actions are in large parts attributable to released paracrine factors rather than direct cell engraftment (Zhou *et al*, 2019). Additional drawbacks of stem cell-based therapies are the requirement for invasive isolation techniques, generally low abundance and high costs for expansion to acquire sufficient material (Ding *et al*, 2023). Therefore, alternative cell types that are more easily available and require less expansive maintenance have moved to the forefront. Peripheral blood mononuclear cells (PBMCs) are distinct cells of the innate and adaptive immune system (Abbas *et al*, 2010). Monocytes, macrophages, natural killer cells, and dendritic cells are derived from myeloid progenitor cells, whereas T- and B-lymphocytes are lymphoid representatives of PBMCs (Abbas *et al.*, 2010). Their immunological functions cover a broad spectrum and encompass direct cellular cytotoxicity, antigen presentation, phagocytosis, production of antibodies and release of paracrine factors including pro- and anti-inflammatory cytokines, growth-factors and chemo-attractants to highlight a few (Abbas *et al.*, 2010). Based on their inert functions PBMCs have been identified as a potent alternative in cell-based therapy. Therefore, the next sections will elaborate on two distinct immunomodulatory strategies involving the cell-based yet cell-free secretome of γ-irradiated peripheral blood mononuclear cells (PBMCsec) and the polyclonal biologic antithymocyte globulin (ATG).

5.1 Antithymocyte globulin

Antithymocyte globulin (ATG) is a mixture of polyclonal antibodies that has been available for treatment of acute T cell mediated rejections in transplantation medicine since the 1980s (Gaber *et al*, 2010). In addition to its use for treatment of steroid resistant T cell mediated rejection reactions ATG is also approved for induction therapy in pre-sensitized transplant recipients for prevention of early rejection reactions, treatment of aplastic anemia and prevention of Graft-versus-host disease in hematopoietic stem cell transplantation (Bamoulid *et al*, 2017; Siddiqui *et al*, 2019). For production of ATG, horses, rabbits or - less frequently - goats are inoculated with cells from human thymus. The resulting fraction of γ - globulins is then purified (Siddiqui *et al.*, 2019; Wechter *et al*, 1979). The obtained antibodies bind a wide range of molecular targets found in T lymphocytes and other immune cells (Popow *et al*, 2013; Rebellato *et al*, 1994). Different formulations with distinctive repertoires of cell surface molecules have been reported for ATG (Bourdage & Hamlin, 1995; Popow *et al.*, 2013; Popow *et al*, 2012). These molecules include various cluster of differentiation molecules present on immunological sub-populations (CD2, CD3, CD4, CD6, CD7, CD8, CD16, CD19, CD20, CD25, CD28, CD30, CD32, CD40) (Bonnefoy-Bérard *et al*, 1991), endothelial adhesion and cell trafficking molecules (LFA-1, VLA-4, ICAM 1-3, CXCR4, CCR7) (Michallet *et al*, 2003), and a more diverse group of molecules involved in inflammation, apoptosis, and proliferation (CD38, CD40, CD80, CD86, CD95, CD126, CD138) (Ashokkumar *et al*, 2015; Mohty, 2007). The immunosuppressive actions of ATG are generally attributed to its T cell depleting properties. There are presently four recognized pathways that account for ATG's ability to deplete T cells. These comprise the following:
(1) Complement-dependent cell cytotoxicity (CDCC) (Ayuk *et al*, 2008)
(2) Opsonization and subsequent clearance via reticuloendothelial uptake (Haidinger *et al*, 2007; Taylor *et al*, 2005)
(3) increased susceptibility to phagocytosis (Taylor *et al.*, 2005)
(4) Activation-induced cell death pathway (AICD) (Ankersmit *et al.*, 2002; Dubey & Nityanand, 2003).
Moreover, suppression of effector function is not solely dependent on lysis of the targeted cell but can also conclude through the downregulation of molecules required for T cell activation, such as the CD3/TCR complex, CD4, CD5, CD6, and CD8. Hence, in addition to its depletive properties ATG also functionally interferes with T cell activation (Haidinger *et al.*, 2007). Furthermore, additional research revealed that ATG's effects on the immune system extend beyond impairment of T cell functions. It was shown that ATG induces apoptosis in naïve activated B-cells and bone-marrow resident plasma cells in vitro (Zand *et al*, 2005), depletes certain subsets of NK-cells while stimulating the remaining NK-cells resulting in degranulation and production of IFN-γ in vitro (Dalle *et al*, 2009) and direct inhibition of

monocyte-derived dendritic cells (Mo-DC) maturation *in vitro* (Roider *et al*, 2016).

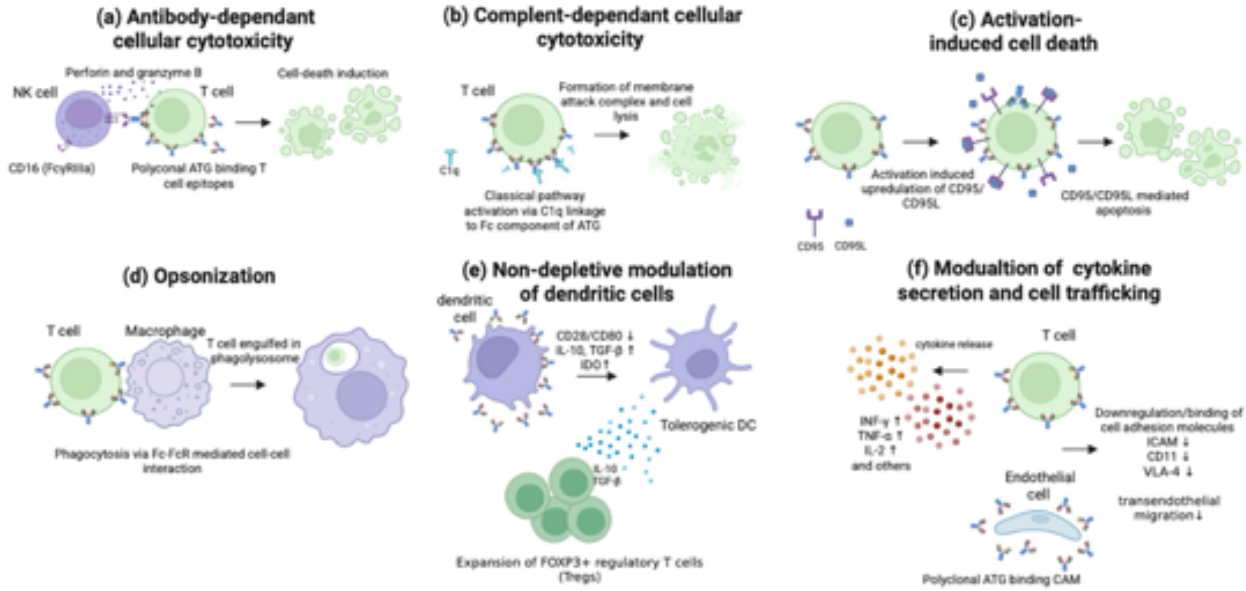

Figure 1. Different modes of action of ATG.
(a) Antibody-dependent cellular cytotoxicity (ADCC): ATG binds T cells and engages FcγRIIIa (CD16) on NK cells which triggers release of cytolytic mediators to promote T-cell death. (b) Complement-dependent cytotoxicity (CDC): ATG-opsonized T cells bind C1q, activating the classical complement pathway and membrane-attack–complex formation resulting in cell lysis. (c) Activation-induced cell death (AICD): ATG-mediated activation upregulates CD95/CD95L (Fas/FasL) on T cells, leading to apoptosis.
(d) Opsonization/phagocytosis: Fc–FcR interactions promote macrophage phagocytosis of ATG-coated T cells into phagolysosomes and consequent lysis. (e) Non-depletive dendritic-cell modulation: ATG skews DCs toward a tolerogenic phenotype with reduced co-stimulation (e.g., CD28/CD80 axis) and increases activity of IL-10, TGF-β, and ID to foster expansion of $FOXP3^{+}$ regulatory T cells (Tregs). (f) Influence on cytokine milieu and cell trafficking control: ATG promotes the release of pro-inflammatory cytokines (e.g., IFN-γ, TNF-α, IL-2) and decreases leukocyte extravasation by binding of cell-adhesion molecules (ICAM, CD11, VLA-4) on T cells and endothelial cells to reduce extravasation. Created in https://BioRender.com

Of note, tissue-protective actions of ATG and other lymphocyte depleting formulations have been investigated in pre-clinical studies other than transplantation medicine. As early as 1970, Földes et al. employed anti-lymphocyte serum to investigate its influence on myocardial infarction in rats (Földes *et al*, 1970). Myocardial tissue from rats exposed to lymphocyte depleting serum displayed diminished inflammatory reactions marked by decreases necrotic tissue. Furthermore, the amelioration in damaged tissue translated to a

reduction in arrhythmogenic events in the rodents (Földes *et al.*, 1970). Similarly, Lichtenauer et al. observed increased levels of cytoprotective and pro-angiogenic paracrine factors in the plasma of ATG-treated whole blood from rats subjected to experimental myocardial infarction (Lichtenauer *et al*, 2012). Strikingly, intravenous administration of ATG showed beneficial short- and long-term effects, which were primarily evidenced by a reduction of the infarction area and preservation of ventricular function (Lichtenauer *et al.*, 2012).

6. Secretome of irradiated peripheral blood mononuclear cells (PBMCsec)

6.1 PBMCsec in acute and chronic myocardial ischemia

Ankersmit et al. were first to report on the beneficial effects of irradiated apoptotic PBMCs in an *in vivo* rat model of acute myocardial infarction (Ankersmit *et al*, 2009). When compared to the control groups – which included culture medium and viable-PBMC - rats injected with the cell suspension of syngeneic irradiated apoptotic PBMCs showed increased presence of $VEGF^+$, $Flk1^+$ and c-kit^+ endothelial progenitor cells in the myocardium after 72 hours. This finding correlated with functional recovery objectified by cardiac output approximating the levels of sham controls, and a significant reduction in infarction size in rats treated with irradiated apoptotic PBMCs at the defined timepoint of six weeks after ligation of the left anterior descending coronary artery (Ankersmit *et al.*, 2009).

Notably, the observed effects were not related to homing of the injected cells into the myocardial tissue but rather resulting from effects mediated by released paracrine factors with chemotactic and pro-angiogenic properties. Elevated protein levels for IL-8, VEGF and MMP9 were detected in supernatants of viable and to an even larger degree that of irradiated apoptotic PBMCs (Ankersmit *et al.*, 2009). Furthermore, co-culturing fibroblasts with supernatants obtained from irradiated apoptotic PBMCs increased mRNA transcripts of MMP9 and CXCL8 after 4 and 24 hours, respectively. Collectively, these data demonstrated that supernatants from irradiated apoptotic PBMCs exert paracrine anti-inflammatory and pro-angiogenic properties *in vitro* and highlighted the beneficial effects of injected irradiated apoptotic PBMCs suspension in an experimental model of AMI in rodents (Ankersmit *et al.*, 2009).

Later research by Lichtenauer et al. employed the same pre-clinal model of experimental AMI to reaffirm that intravenous or intramyocardial injection of irradiated apoptotic PBMCs cell suspensions positively modifies the composition of cardiac scar tissue while also attenuating myocardial remodeling and preserving left ventricular systolic function (Lichtenauer *et al*, 2011a).

Based on their observations Ankersmit et al. developed a cell-based yet cell-free therapeutic consisting of paracrine factors secreted and released by γ-irradiated apoptotic PBMCs (hereinafter referred to APOSEC™ or PBMCsec) (Lichtenauer *et al*, 2011b). In a rodent and porcine model of experimental AMI and closed chest reperfusion AMI, respectively, a single intravenous injection of PBMCsec resulted in decreased myocardial remodeling marked by improved left ventricular systolic ejection fraction, increased cardiac output, and decreased myocardial scar tissue formation (Lichtenauer *et al.*, 2011b). Beyond that, addition of PBMCsec to human primary cardiomyocytes conferred cyto-protection by activation of

transcription factors associated with pro-survival signaling including AKT, Erk1/2, CREB, c-Jun (Lichtenauer *et al.*, 2011b). Furthermore, PBMCsec revealed greater concentrations of IL-8, GRO-alpha, ENA-78, RANTES, sICAM-1, MIF, VEGF, IL-1ra, and IL-16 in comparison to the supernatant of non-irradiated PBMCs revealing profound differences in soluble factor profiles being a crucial determinant for PBMCsec and its effects (Lichtenauer *et al.*, 2011b). In an extension of the previously described porcine closed chest reperfusion model for AMI, intravenous injection of PBMCsec resulted in smaller regions of microvascular obstruction (MVO) during follow-up examination assessed by magnetic resonance imaging (Hoetzenecker *et al*, 2012). Cardiac catheterization revealed significantly reduced corrected thrombolysis in myocardial infarction (TIMI) frame counts in animals treated with PBMCsec and further substantiated this finding (Hoetzenecker *et al.*, 2012). Moreover, lower event rates for ventricular arrhythmias were recorder in the group receiving PBMCsec. Additionally, animals injected with PBMCsec displayed improved ventricular contraction capability with resolution of ST-segment elevation in four out of six as opposed to one out of seven animals in the control group (Hoetzenecker *et al.*, 2012). Pig sera obtained at defined timepoints after AMI revealed reduced levels of sCD40L, TSP-1, soluble P-selectin, and PF-4, representing soluble markers of platelet activation and contributors to MVO. The *in vivo* observations were followed up by *in vitro* trials involving porcine and human derived platelets. Addition of PBMCsec inhibited platelet aggregation *in vitro* and was linked to an increase activation of vasodilator-stimulated phosphoprotein (VASP), a known inhibitor of platelet activation(Aradi *et al*, 2010; Hoetzenecker *et al.*, 2012).

In addition to the effects in animal studies of acute myocardial infarction, beneficial influences of PBMCsec were also evidenced in models of chronic ischemia following myocardial infarction. (Pavo *et al*, 2014) Assessment of myocardial regeneration in porcine chronic post-myocardial infarction linked intramyocardial injection of PBMCsec to reduced myocardial lesion size and increased contractile performance (Pavo *et al.*, 2014). On a microscopic level tissue obtained from animals treated with PBMCsec displayed enhanced angiogenesis, higher vascular density, and increased homing of c-kit^{+} endogenous stem cells in the infarcted region as well as adjacent myocardium (peri-infarction region or border zone) (Pavo *et al.*, 2014). Gene profiling studies of PBMCsec treated cardiac regions revealed differences in gene expression for gene sets associated with angiogenesis, inflammation, apoptosis, and lipid-metabolism in the infarction zone, border zone and remote myocardium (Pavo *et al.*, 2014). Gene set enrichment analysis revealed a significant overrepresentation of biological processes associated with striated muscle cell development, heart contraction and myofibril assembly in the gene set of differentially upregulated genes in infarcted myocardium of the PBMCsec treated animals. In contrast, immune system processes including migration and

chemotaxis of immune cell subsets and T-cell proliferation were enriched when computing the differentially downregulated genes (Mildner *et al*, 2022).
Regarding the border zone, processes associated with negative regulation of blood coagulation, angiogenesis in wound healing and positive regulation of proliferation were amongst pathways enriched by the differentially upregulated genes induced by PBMCsec (Mildner *et al.*, 2022).
Furthermore, PBMCsec-mediated alterations in gene expression were not restricted to the myocardium but also present in distant organs such as liver and spleen (Mildner *et al.*, 2022). Systemic effects of PBMCsec on the transcriptional profile of the liver revealed an overrepresentation of biological pathways associated with acute-phase responses and antimicrobial humeral responses, metabolic activity, and negative regulation of endopeptidase activity (Mildner *et al.*, 2022). Taken together, these findings provided strong evidence for systemic effects of PBMCsec extending beyond affected tissue at risk and further underlining its broad therapeutic applicability.
With a growing body of evidence suggesting favorable influences of PBMCsec in pro-angiogenetic and immunological processes, Hötzenecker et al. investigated the immunomodulatory effects of PBMCsec in a murine model of experimental autoimmune myocarditis (Hoetzenecker *et al*, 2015). Intraperitoneal injection of syngeneic PBMCsec resulted in diminished lymphocyte infiltration, almost completely abrogating myocarditis in the examined animals (Hoetzenecker *et al.*, 2015). Mechanistically, application of PBMCsec induced caspase-8-dependent apoptosis in $CD4^+$-T-cells an impaired their proliferative capacity after stimulation (Hoetzenecker *et al.*, 2015). Identification of the functional components and broad spectrum of therapeutic applicability of PBMCsec in tissue repair beyond myocardial damage were further examined in ensuing studies (Beer *et al*, 2016).

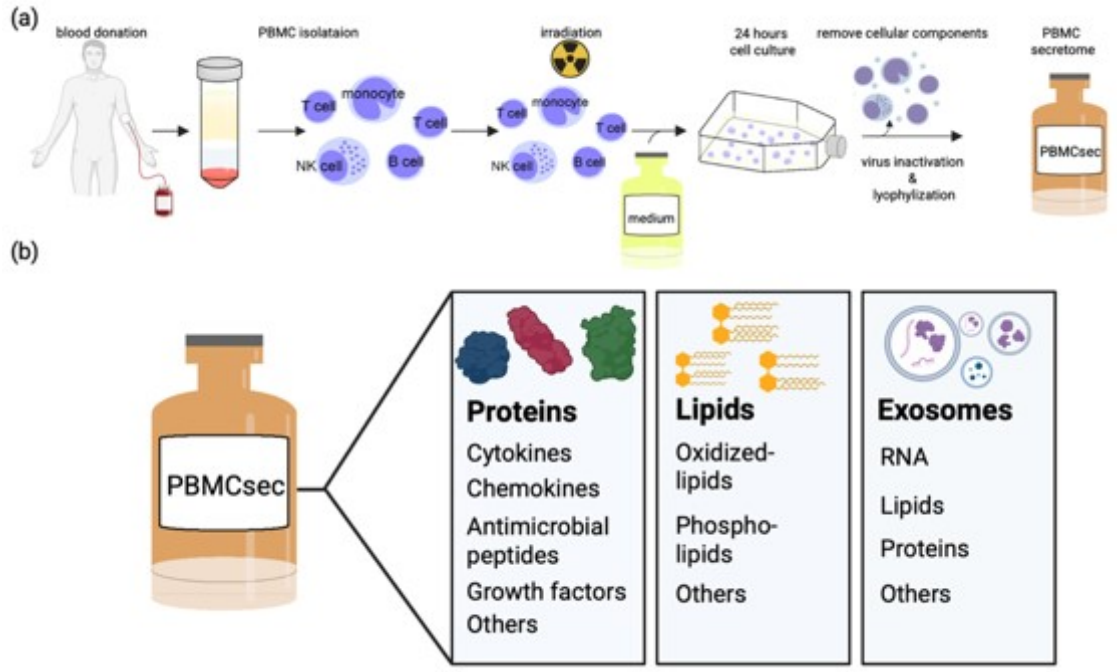

Figure 2. Generation and composition of PBMCsec.
(a) Whole blood is collected from healthy donors. Isolated PBMCs are irradiated to induce programmed cell death and subsequently cultured for 24h. Cellular removal is followed by viral inactivation and lyophilization to yield the cell-free PBMC secretome (PBMCsec) for reconstitution. (b) PBMCsec contains various constituents comprising proteins, lipids and exosomes/extracellular vesicles altogether aiding in its multimodal actions. Created in https://BioRender.com

6.2 PBMCsec in central and peripheral nervous system injury

In a rat middle cerebral artery occlusion model, Altmann et al. investigated the effects of rat- and human-PBMCsec on ischemic lesion volumes (Altmann *et al*, 2014). Rats were treated intraperitoneally with a defined dosage of syngeneic or human PBMCsec at set time-points of either 40 minutes or 24 hours after permanent occlusion of the middle cerebral artery. Reduction of focal lesion size and improvement in motor functions assessed by a seven-point neurological scoring system was registered in animals receiving PBMCsec irrespective of source (Altmann *et al.*, 2014). Moreover, human PBMCsec activated CREB, Akt, c-Jun, and Erk1/2 signaling cascades in primary Schwann cells and astrocytes cultured with suggesting promotion of cyto-protection (Altmann *et al.*, 2014).
The therapeutic effects of PBMCsec are not confined to vascular insults to the central nervous system. Instead, Haider et al. demonstrated significant improvements in an experimental model of traumatic spinal cord injury in rats where administration of PBMCsec resulted in volume reduction of inflicted lesion, which was additionally marked by diminished

axonal damage and increased vessel density (Haider *et al*, 2015). Additionally, PBMCsec enhanced recruitment of CD68+ cells with concomitant reduction of oxidative stress (Haider *et al.*, 2015). These histopathological findings translated to a significant difference in functional recovery of motor functions favoring animals treated with PBMCsec when compared to controls (Haider *et al.*, 2015).

6.3 PBMCsec in wound healing and scar formation

The effects of PBMCsec are not limited to intravenous application but have also been demonstrated to achieve remarkable results when topically administered. Mildner et al. were able to demonstrate that emulsions containing PBMCsec accelerated wound healing in a rodent model of punch wound in C57BL/6J mice (Mildner *et al*, 2013). Daily administration over the course of three days after initial wounding resulted in accelerated wound closure and smaller wound gap distance with significantly improved re-epithelialization in animals treated with emulsions containing PBMCsec. Final read-outs were assessed at day 6 post wounding. Furthermore, addition of PBMCsec to cultured primary keratinocytes and fibroblast significantly promoted cell migration in an *in vitro* scratch assay. Of note, no significant changes in cell cycle progression were detected following treatment of the respective cell types with PBMCsec (Mildner *et al.*, 2013). This notion further asserts that the effect is based on cell migration rather than induction of proliferation in primary keratinocytes and fibroblasts. Beyond that, topical treatment with PBMCsec significantly increased blood vessel density as well as percentage of $CD31^+$ cells altogether suggesting enhanced angiogenesis, which represents a crucial step in wound healing (Mildner *et al.*, 2013). Additionally, PBMCsec improved tube formation in an *in vitro* endothelial cell tube formation assay while no effects were observed by the control media (Mildner *et al.*, 2013).
To uncover the underlying mechanism driving these effects, involvement of a broad panel of signaling pathways associated with cell migration, proliferation and survival was explored. PBMCsec led to phosphorylation of CREB, Erk1/2, c-Jun, Akt and Hsp27 in human primary keratinocytes, while Erk1/2, c-Jun, Akt and Hsp27 in primary dermal fibroblasts. Addition of PBMCsec to dermal microvasculature endothelial cells resulted in activation of CREB, c-JUN and Hsp27, respectively (Mildner *et al.*, 2013). Hacker et al. followed up on these findings and evaluated PBMCsec for treatment of full thickness burn injuries in a porcine model (Hacker *et al*, 2016). When compared to controls lesions treated with PBMCsec displayed significant improvements in epidermal thickness, more advanced epidermal differentiation, less stiffness, better elasticity and increased presence of $CD31^+$ cells, falling in line with previously reported findings (Hacker *et al.*, 2016; Mildner *et al.*, 2013). Additional findings included a trend towards diminished presence of mast cells and an increase in $ASMA^+$ cells in the dermal layers of affected tissues. However, it is important to emphasize that these

differences were most pronounced when comparing PBMCsec treatment to NaCl and carrier medium while no statistically significant difference between PBMCsec and the secretome of non-irradiated PBMCs were noted (Hacker *et al.*, 2016). Additional beneficial impacts of PBMCsec on the process of wound healing were highlighted in a murine model epigastric flap model where a single dosage of PBMCsec together with a fibrin sealant during surgery significantly reduced tissue necrosis of the epigastric flap (Hacker *et al*, 2021).
Robust antimicrobial defense mechanisms are crucial for sufficient wound healing. (Kasiri *et al*, 2016) While the secretome of non-stressed PBMCs exerts antimicrobial activity against bacteria such as Pseudomonas aeruginosa, Escherichia coli, and Staphylococcus aureus, PBMCsec had been demonstrated to exert superior inhibitory effects on these bacteria and significantly reduced their growth and thus aiding wound healing (Kasiri *et al.*, 2016).
While these studies aimed to elucidate the impact of PBMCsec on the early phase of wound healing, they did not provide any insights into its influence on scar formation and impact on preexisting scar tissue. This exact question was addressed by Vorstandlechner et al. in a murine full-thickness skin wound model and *ex vivo* human scars (Vorstandlechner *et al*, 2023). The experimental setup involved topical and intradermal application of PBMCsec to freshly inflicted wounds and formed scar tissue, respectively. Topical administration of a PBMCsec containing ointment reduced wound size significantly. Histological assessment of scar tissue revealed more loose arrangement and reduced fiber density in samples of intradermally injected with PBMCsec (Vorstandlechner *et al.*, 2023). Exploration of the single cellular transcriptional changes mediated by PBMCsec in murine scars revealed alterations in gene expression of various target genes involved in pro-fibrotic processes and tissue remodeling (Vorstandlechner *et al.*, 2023). Amongst those, genes involved in extracellular matrix formation including collagens, proteoglycans and glycoproteins were mostly downregulated (Vorstandlechner *et al.*, 2023). Similar findings were evident in human scar that had been treated with PBMCsec *ex vivo* (Vorstandlechner *et al.*, 2023). These effects were in part attributable to the inhibitory effect of PBMCsec on TGF-β-mediated myofibroblast differentiation and prevention of elastin breakdown (Vorstandlechner *et al.*, 2023).

6.4 Immunomodulatory effects of PBMCsec

In addition to its well documented effects on wound healing and scar formation, Laggner et al. demonstrated the inhibitory effects of PBMCsec on inflammation and cell infiltration in dendritic cell (DC)-mediated skin inflammation in mice (Laggner *et al*, 2020a). Differentiation of monocyte derived DCs was abrogated in the presence of PBMCsec a d resulted in lowered expression of DC markers including MHC class II molecules, CD11c and CD1a. Alteration in the differentiation process secondary to exposure to PBMCsec impaired the capability of monocyte-derived DC to prime naïve CD4+ T-cells into committed TH1 and TH2 cells (Laggner *et al.*, 2020a). Furthermore, PBMCsec impacted maturation of fully differentiated monocyte-derived DC by decreasing effector functions such as lipopolysaccharide-induced cytokine secretion, DC-mediated immune cell proliferation as well as antigen uptake (Laggner *et al.*, 2020a). Dissection of the substance specific components present in PBMCsec revealed a central role for PBMCsec-derived lipids in the promotion of the described immunomodulatory changes (Laggner *et al.*, 2020a).
Investigation on the effects of PBMCsec on mast cells and basophils in the context of IgE-mediated hypersensitivity revealed favorable outcomes (Laggner *et al*, 2022). Topical application of PBMCsec to mouse ears significantly reduced experimentally induced mast cell degranulation. Transcriptional analysis showed downregulation of genes associated with Fc-receptor signaling and immune cell degranulation. Similarly, treatment of activated human dermal mast cells *in vitro* with PBMCsec strongly reduced anaphylactic mediator release triggered by α-IgE and compound 48/80. Furthermore, PBMCsec treatment reduced allergen-induced activation of basophils from allergic donors. Accordingly, transcriptome analysis of these PBMCsec-treated basophils revealed extensive downregulation of genes involved in Fc-receptor signaling and cell degranulation. The study identified lipids within the PBMCsec as the main component supporting these immunomodulatory effects (Laggner *et al.*, 2022).
Klas et al. expanded on the immunomodulatory properties on PBMCsec by investigating the influences of PBMCsec on experimentally induced formation of neutrophil extracellular traps (NETs) in human neutrophils *ex vivo* (Klas *et al*, 2022). Treatment of neutrophils with PBMCsec prior to activation using ionomycin resulted in an inhibition of NETs formation. Mechanistically, PBMCs reduced intracellular production of reactive oxygen species and inhibited PAD4 activity by approximately 40% when compared to vehicle control. Interestingly, assessment of the inhibitory effects mediated by the major PBMCsec component classes individually – namely proteins, lipids, DNA contents and extracellular vesicles – showed only partial reduction in NET formation, thus suggesting a synergistic effect underlying the complex interplay of all biological fractions present in PBMCsec (Klas *et al.*, 2022).

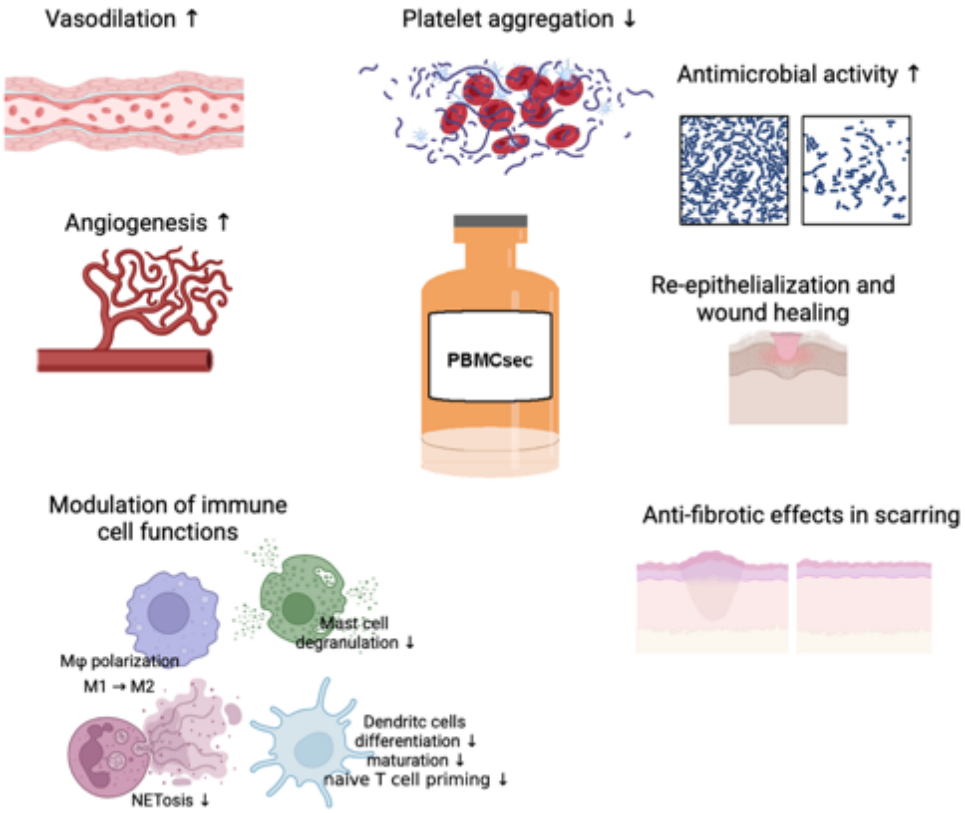

Figure 3. Multimodal actions of PBMCsec to support tissue-repair.
PBMCsec promotes vasodilation and angiogenesis, reduces platelet aggregation, and displays antimicrobial activity. In the setting of wound healing PBMCsec accelerates re-epithelialization and promotes anti-fibrotic effects to influence scar formation. PBMCsec also modulates immune cell functions by decreasing mast-cell degranulation, promoting macrophage polarization from pro-inflammatory M1 to pro-resolving M2. Additonally, PBMCsec reduces NETosis and affects dendritic-cell functions by inhibiting differentiation/maturation along with naïve T-cell priming. ↑ indicates increase; ↓ indicates decrease. Created in https://BioRender.com

6.5 Transition to clinical trials – MARSYAS I & MARSYAS II

The reported effects of PBMCsec in models of induced tissue injuries ranging from attenuation of inflammation to improvement of wound healing paved the way for the first in man trial for PBMCsec (Simader *et al*, 2017). The MARSYAS I trial was a prospective, randomized, placebo-controlled, single-center, double-blind phase I trial investigating the safety and tolerability of topically administered autologous PBMCsec in dermal wounds (Simader *et al.*, 2017). Study participants were volunteer healthy male individuals. Two separate 4mm punch biopsy wounds were placed on their upper arm. Following randomization one wound was either treated with low (concentrated at $12.5x10^6$ PBMCs/ml) or high (concentrated at $25x10^6$ PBMCs/ml) dose PBMCsec resuspended in NuGel Hydrogel while the other wound received placebo. Cell medium served as placebo control. The primary endpoint of safety and tolerability was met. The adverse events reported and captured during follow up were all characterized as mild and unlikely to be related to the treatment. As for the secondary outcome measure, which assessed between groups comparison in wound closure, no significant differences were observed (Simader *et al.*, 2017).

This trial led to the initiation of the follow-up trial MARSYAS II (Gugerell *et al*, 2021). This was a Phase IIa randomized, multi-center, placebo-controlled, double-blind trial evaluating the safety and efficacy of PBMCsec (APOSEC™; referenced as APO-2 in the Clinical Study Report; EudraCT No.: 2018-001653-27) in patients with diabetic foot ulcers (DFU). The primary objective was to assess the dose-dependent clinical efficacy of PBMCsec in promoting wound healing by comparing three dosages to placebo. Secondary objectives included evaluating the safety and tolerability of PBMCsec and its effects on additional wound healing outcomes. The trial was conducted at multiple centers in Austria, Germany, the Czech Republic, and Poland between November 2020 and December 2023. An initial safety lead-in phase enrolled 12 patients that were treated with investigational product and placebo controls for 4 weeks. No safety concerns were evident allowing subsequent conclusion of the main study. Patients were randomized to one of three PBMCsec dose groups – low (12.5 U/mL), medium (25.0 U/mL), or high (50.0 U/mL) – or placebo. Ulcer size was used for stratification of trial participants. Treatments were administered topically three times per week for a total of 4 weeks and were added on top of standard measures for wound care provided to all patients. After this period patients were followed for an additional 8 weeks after treatment to assess wound healing durability. A total of 122 patients with chronic DFU were enrolled and randomized into the four study arms.

The primary endpoint for efficacy was prespecified as the percentage reduction in wound area from baseline after the 4-week treatment period. Secondary endpoints included various

measures of wound healing efficacy and patient outcomes. Amongst those the proportion of patients achieving >50% ulcer area reduction after 4 weeks, time to complete wound closure, rate of complete wound closure by 12 weeks of follow-up and ulcer recurrence rate after closure were specified. Additional secondary endpoints involved clinical assessments of peripheral neuropathy and patient-reported outcomes including wound pain and wound-specific quality of life. Safety endpoints were also defined, including the incidence of adverse events (AEs), any treatment-related or serious adverse events, and changes in vital signs, laboratory parameters, and physical examinations.

The primary endpoint analysis showed no statistically significant differences in percent wound area reduction between any PBMCsec dose group and placebo after 4 weeks (all $p>0.05$). However, numerical trends favored PBMCsec. The low-dose (12.5 U/mL) and high-dose (50 U/mL) groups achieved greater mean wound area reductions (approximately 59% and 47%, respectively) than the placebo group (29%). Secondary healing outcomes also suggested improved efficacy with PBMCsec. Notably, 48% of patients in the high-dose group attained ≥80% wound closure by week 4, compared to 20% in the placebo group with a post-hoc analysis revealing a statistically significant result with $p<0.05$.

By the 12-week follow-up, complete ulcer closure rates remained higher in the PBMCsec arms than in placebo. In a subgroup analysis excluding very small ulcers, the medium-dose PBMCsec group achieved almost double the closure rate of placebo (35% vs 18% by week 12). A post-hoc analysis limited to patients with baseline wound area ≥0.8 cm^2 confirmed the overall positive healing trends seen with PBMCsec treatment. Regarding safety endpoint the MARSYAS II trial revealed generally good tolerability for PBMCsec with a safety profile comparable to placebo. The incidence of treatment-emergent adverse events (TEAEs) in the PBMCsec groups ranged from about 35–47%, comparable to 45% in the placebo group, and showed no clear dose-related pattern. No deaths occurred during the study, and all 11 serious adverse events reported were considered unrelated to the treatment. Overall, the safety data indicate that APO-2 did not elicit any concerning adverse effects in this patient population.

Altogether, the MARSYAS II trial indicates that PBMCsec may improve wound healing in diabetic foot ulcers, though no statistically significant benefit over placebo was observed after 4 weeks of treatment. The hydrogel-based placebo likely had inherent healing effects, potentially obscuring PBMCsecs efficacy. Future studies should compare PBMCsec to standard care alone and extend the treatment duration to at least 12 weeks. Given its favorable safety and encouraging trends, PBMCsec warrants further evaluation in larger trials with adjusted designs (Clinical Study Report; EudraCT No.: 2018-001653-27).

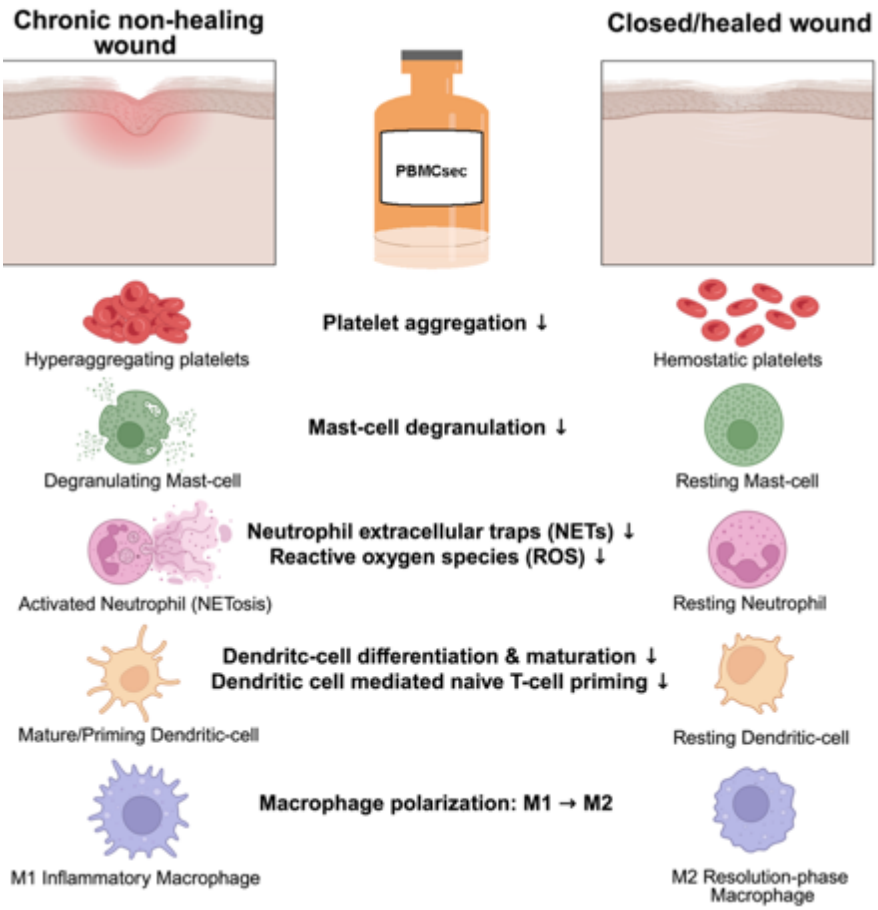

Figure 4.: Immunomodulatory effects of PBMCsec promoting resolution of chronic wounds. Created in https://BioRender.com

Experimental model	Species	Route of administration	Source matarial	Observerd effects	References
AMI	Rat	i.v.	Irradiated PBMC suspension (syngeneic)	enhanced homing of endothelial progenitor cells reduction in infarction size improvement in post AMI remodeling	(Ankersmit *et al.*, 2009)
AMI	Pig	i.v.	Irradiated PBMC suspension (syngeneic)	reduction of infarction size and an improvement of post AMI remodeling preservation of left ventricular function increases homing of regenerative cells increased elastin expression with favorable composition of cardiac scar tissue	(Lichtenauer *et al.*, 2011a)
AMI	Pig	i.v.	PBMCsec (syngeneic)	reduction of scar tissue formation improved cardiac function (LVEF, CO) upregulation of pro-survival pathways in primary cardiac myocytes	(Lichtenauer *et al.*, 2011b)
AMI	Pig	i.v.	PBMCsec (syngeneic)	Reduction of platelet activation markers Increased vasodilatory capacity via p-eNOS and iNOS activation	(Hoetzenecker *et al.*, 2012)
Full thickness skin wound	Mice	topical	PBMCsec	Accelerated wound healing with augmented re-epithelialization Promoted cell migration in keratinocytes and fibroblasts Increased blood vessel density	(Mildner *et al.*, 2013)
Stroke	Rat	i.p.	PBMCsec	Reduction of focal lesion size Improvement in motor functions	(Altmann *et al.*, 2014)
Chronic heart failure	Pig	i.v./ intra-myocardial	PBMCsec	reduced myocardial lesion size increased contractile performance enhanced angiogenesis	(Pavo *et al.*, 2014)
SCI	Rat	i.p.	PBMCsec	Reduction of lesion size Decreased axonal damage Improved vessel density	(Haider *et al.*, 2015)
EAM	mice	i.p.	PBMCsec	Resolution of acute inflammation	(Hoetzenecker *et al.*, 2015)

Burn injury	Pig	topical	PBMCsec (human, GMP)	Improvements epidermal thickness diminished presence of mast cells and an increase in ASMA+ cells increased presence of CD31+ cells	(Hacker *et al.*, 2016)
Dermal wound	Human (Phase I)	topical	PBMCsec (human, GMP)	Safety and tolerability (ClinicalTrials.gov NCT02284360)	(Simader *et al.*, 2017)
Dendritc-cell mediated skin inflammation	Mice	topical	PBMCsec (human, GMP)	Abrogation of monocyte-derived DC differentiation Inhibition of DC-mediated immune cell proliferation as well as antigen uptake	(Laggner *et al.*, 2020a)
Diabetic foot ulcer	Human (Phase I/II)	topical	PBMCsec (human, GMP)	Safety and clinical efficacy (ClinicalTrials.gov NCT04277598)	(Gugerell *et al.*, 2021)
Epigastric flap model	Rat	i.v.	PBMCsec (human, GMP)	Improved epigastric flap healing with decreased tissue necrosis	(Hacker *et al.*, 2021)
IgE-mediated Hypersensitivity	Mice	topical	PBMCsec (human, GMP)	Mitigation of compound 48/80 and IgE-induced degranulation of mast cells downregulation of genes involved in immune cell degranulation and Fc-receptor signalling Attenuation of allergen-driven activation of basophils from allergic individuals	(Laggner *et al.*, 2022)
NETosis	Human granulocytes	*ex vivo* co-incubation	PBMCsec (human, GM)	Inhibition of NETosis Decrease of ROS production Decrease in PAD4 activity	(Klas *et al.*, 2022)
Scar formation	Mice	topical / intradermal injection	PBMCsec (human, GMP)	Prevention of TGFβ-mediated myofibroblast differentiation Attenuation of abundant elastin expression with non-canonical signaling inhibition Inhibition of TGFβ-induced breakdown of elastic fibers	(Vorstandlechner *et al.*, 2023)

Table 1: PBMCsec in preclinical and clinical models of tissue repair and immunomodulation

7. Aims of this thesis

ATG and PBMCsec are two immunomodulatory agents with distinctive mechanistic profile. The following aims are addressed in this thesis.

Aim 1:
Characterize novel immunomodulatory effects of ATG that extend beyond its depletive mechanisms and contribute to paracrine factor mediated modulation of peripheral tolerance.

Aim 2:
Identify the transcriptional and functional changes induced by PBMCsec in human whole blood with identification of regulated processes implicated for tissue regeneration

Aim 3:
Comparison of the distinct yet converging immunomodulatory principles of ATG and PBMCsec and evaluation of their potential as indirect modulators of immune function through reprogramming of immunological functions and to assess their broader implications for immunotherapy and regenerative medicine.

CHAPTER TWO: RESULTS

2. Prologue

Cytoprotective and immunomodulatory functions of PBMCsec and its subfractions on specific immune and non-immune cell types have been elucidate in preceding pre-clinical and clinical studies. The objective of this thesis was to assess the transcriptional and functional changes PBMCsec exerts on immune cells from human whole blood. Thereby, we aimed to add significant novelty to the already broad mechanistic repertoire of PBMCsec and to expand the field for future therapeutic applications.

2.1 First Publication

pharmaceutics

MDPI

Article

Paracrine Factors of Stressed Peripheral Blood Mononuclear Cells Activate Proangiogenic and Anti-Proteolytic Processes in Whole Blood Cells and Protect the Endothelial Barrier

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Abstract: Tissue-regenerative properties have been attributed to secreted paracrine factors derived from stem cells and other cell types. In particular, the secretome of γ-irradiated peripheral blood mononuclear cells (PBMCsec) has been shown to possess high tissue-regenerative and proangiogenic capacities in a variety of preclinical studies. In light of future therapeutic intravenous applications of PBMCsec, we investigated the possible effects of PBMCsec on white blood cells and endothelial cells lining the vasculature. To identify changes in the transcriptional profile, whole blood was drawn from healthy individuals and stimulated with PBMCsec for 8 h ex vivo before further processing for single-cell RNA sequencing. PBMCsec significantly altered the gene signature of granulocytes (17 genes), T-cells (45 genes), B-cells (72 genes), and, most prominently, monocytes (322 genes). We detected a strong upregulation of several tissue-regenerative and proangiogenic cyto- and chemokines in monocytes, including *VEGFA*, *CXCL1*, and *CXCL5*. Intriguingly, inhibitors of endopeptidase activity, such as *SERPINB2*, were also strongly induced. Measurement of the trans-endothelial electrical resistance of primary human microvascular endothelial cells revealed a strong barrier-protective effect of PBMCsec after barrier disruption. Together, we show that PBMCsec induces angiogenic and proteolytic processes in the blood and is able to attenuate endothelial barrier damage. These regenerative properties suggest that systemic application of PBMCsec might be a promising novel strategy to restore damaged organs.

Keywords: regenerative medicine; cell-free secretomes; paracrine factors; single-cell RNA sequencing; serine protease inhibitor; endothelial barrier

1. Introduction

Central goals in modern regenerative medicine are the repair of injured tissues and organs along with restoration of their innate functions [1]. Cell-based therapies have made tangible progress in this field over the past decades [2]. Mesenchymal stem cells (MSC) possess high capacities for self-renewal and differentiation, which makes them a highly attractive option to promote tissue regeneration. However, in spite of encouraging pre-clinical data, most of the first-in-men clinical trials failed to show effectivity in patients [3,4]. Beyond that, it became increasingly apparent that released paracrine factors, rather than engraftment and differentiation of applied stem cells, are crucial for most of the beneficial

biological effects and, thus, predominantly contribute to the observed tissue-regenerative effects [5–8]. In addition, stem-cell secretome-based therapies bear considerable limitations, including the need for invasive procedures for isolation, low abundance, and high costs for expansion and preservation. This highlights the need for alternative sources [9].

Recently, several studies suggested peripheral blood mononuclear cells (PBMCs) as a valuable alternative source to MSCs [10,11]. In addition, γ-irradiation of PBMCs has been shown to promote the production and release of soluble factors and to exert tissue protection [10]. In-depth functional analyses of the secretome of γ-irradiated PBMCs (PBMCsec) revealed a vast array of proteins, lipids, and extracellular vesicles as the main biological constituents and confirmed that the presence of all fractions is required in order for PBMCsec to exert its biological effects to full capacity [12,13]. The modes of action via which PBMCsec exerts its beneficial effects range from promotion of angiogenesis [12], antimicrobial activity [14], cytoprotection [15], immunomodulation [16], improvement of re-epithelization [17] to vasodilation and the inhibition of platelet aggregation [18]. More recently, Laggner and colleagues were able to demonstrate a decrease of dendritic cell-mediated skin inflammation in a murine model of contact hypersensitivity, as well as inhibition of basophil and mast cell degranulation after treatment with PBMCsec, further expanding the broad spectrum of possible clinical implications of this investigational medicinal product [19,20]. Beneficial effects of PBMCsec have thus far been examined in numerous preclinical setups of experimental tissue damage, including acute myocardial infarction, autoimmune myocarditis, stroke, and spinal cord injury (for a review, see [21]). Topical administration of PBMCsec enhanced wound healing in a murine full-thickness skin model [17] and improved tissue survival in a rodent epigastric flap model [22]. Similar results were observed in a porcine model of burn injury, where the application of PBMCsec markedly improved epidermal thickness after injury [23]. In addition, beneficial effects of PBMCsec were also observed after intraperitoneal or intravenous administration. PBMCsec decreased the affected area and improved neurological outcome in rodent models of cerebral ischemia and acute spinal cord injury after systemic application [24,25]. Furthermore, PBMCsec ameliorated myocardial damage, improved overall cardiac performance, and promoted cytoprotection of cardiomyocytes in rodent and porcine models of acute myocardial infarction (AMI) [10,15]. Interestingly, transcriptional changes were not restricted to the infarcted myocardium and the circumjacent heart tissue but also detected in the liver and the spleen, suggesting a systemic effect of PBMCsec beyond the site of injury [26].

For the treatment of cardiovascular pathologies caused by thromboembolic occlusion of arterial blood flow such as acute myocardial infarction and ischemic stroke, the rapid interventional re-establishment of vessel perfusion still remains the first-line therapy as it maximizes the rescue of ischemic tissue and, thus, decisively determines the primary outcome in affected patients [27]. However, increasing emphasis is attributed to the attenuation of damages secondary to reperfusion injury, as it represents a considerable risk factor for long-term tissue functions [28]. During extended periods of oxygen deprivation, cells suffer from intracellular calcium overload [29] and disturbed mitochondrial energy production [30], which ultimately lead to cell death and the release of inflammatory mediators and reactive oxygen species [31]. In turn, inflammatory cells infiltrate the damaged area and release proinflammatory cyto- and chemokines. In addition, neutrophils release serine proteases [32,33], which contribute to endothelial barrier dysfunction, further enhancing vascular injury in small arterial blood vessels and downstream capillaries [34]. As a result, large amounts of intravascular fluids along with the damaging mediators diffuse into the interstitial space to cause further damage [34].

Since PBMCsec has shown tissue-regenerative properties in several animal models and there is an urgent need for novel systemic tissue-regenerative therapeutic interventions, we investigated the effects of PBMCsec on white blood cells and on the endothelial barrier function.

2. Materials and Methods

2.1. Ethics Statement

This study was conducted in accordance with the Declaration of Helsinki and local regulations. Blood samples were obtained from healthy volunteers who had given their consent to donate. Use of primary HUVECs, primary DMECs, and blood samples was approved by the Institutional Review Board of the Medical University of Vienna (Ethics committee votes: 1280/2015, 1539/2017, and 1621/2020). All donors provided written informed consent.

2.2. Generation of PBMCsec

Isolation of PBMCs and generation of PBMCsec have previously been described in detail [35], and a graphical overview is given in Figure 1A. In brief, PBMCs of volunteer blood donors aged 18–45 years were enriched by Ficoll-Paque PLUS (GE Healthcare, Chicago, IL, USA) density centrifugation, diluted to a concentration of 2.5×10^7 cells/mL in CellGenix granulocyte–monocyte progenitor dendritic cell medium (CellGenix, Freiburg, Germany) and exposed to 60 Gy cesium-137 γ-irradiation (IBL 437C, Isotopen Diagnostik CIS GmbH, Dreieich, Germany). Following 24 h of incubation, cells and cellular debris were removed by centrifugation at 800× *g* for 15 min, and supernatants were passed through a 0.2 mm filter. The cell-free secretome generated by 2.5×10^7 cells/mL corresponds to 25 units/mL PBMCsec. Next, methylene blue treatment was performed for viral clearance [35]. Secretomes were lyophilized, terminally sterilized by high-dosage-irradiation (Gammatron 1500, UKEM60Co irradiator with a maximum capacity of 1.5 MCi), and cryopreserved. Lyophilized compounds were reconstituted in 0.9% NaCl (B. Braun Melsungen AG, Melsungen, Germany) to the desired concentrations.

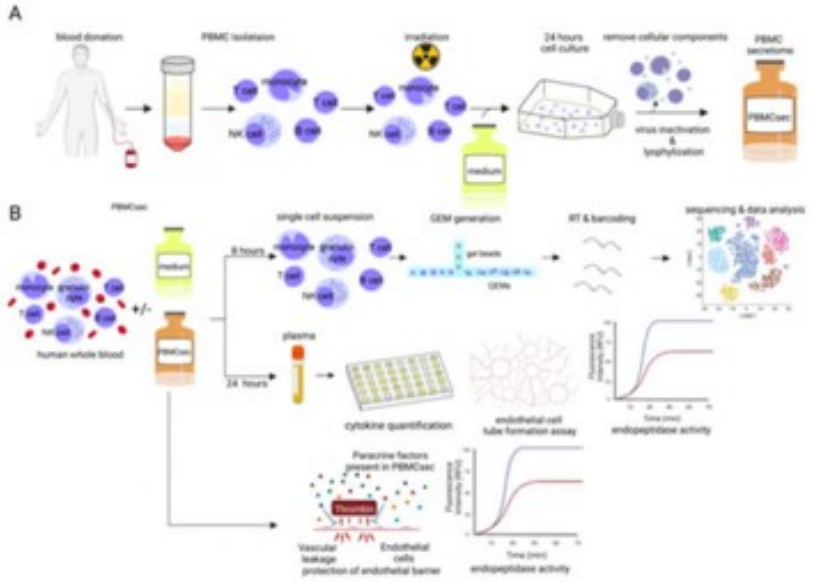

Figure 1. Experimental overview (**A**) Isolation of PBMCs from healthy donors and subsequent procedures necessary for the generation of PBMCsec. (**B**) The experimental setup involved two branches. In order to investigate pharmacodynamics effects of PBMCsec, human whole blood cells were treated with PBMCsec or left untreated for 8 h prior to further processing them for single-cell RNA sequencing. Plasma was collected from PBMCsec-treated whole blood and controls after 24 h of incubation and analyzed in a series of functional assays. In addition, we evaluated effects mediated directly by PBMCsec on endopeptidase activity and endothelial barrier protection. This figure was generated using Biorender.com (accessed on 6 June 2022).

2.3. Preparation of Single-Cell Suspension of Human Whole Blood

For scRNA-seq, heparinized human whole blood was drawn from two age-matched male donors (age donor 1: 29 years; age donor 2: 29 years). A total of 3 mL of whole blood was either treated with PBMCsec (GMP APOSEC lot number: A00918399135; diluted in 0.9% NaCl; final concentration: 12.5 units/mL) or left untreated. Samples were incubated at 37 °C for 8 h. Red blood cells were removed by Red Blood Cell Lysis Buffer (Abcam, Cambridge, MA, USA). Cells were then washed twice with PBS containing 0.04% bovine serum albumin (BSA) and sequentially passed through 100 and 40 µm cell strainers. Using the LUNA-FL Dual Fluorescence Cell Counter (BioCat, Heidelberg, Germany) and the Acridine Orange/Propidium Iodide Cell Viability Kit (Logos Biosystems, Gyeonggi-do, South Korea), samples were set at a concentration of 1×10^6 cells/mL and displayed a viability above 90%.

2.4. Dermal Microvascular Endothelial Cell Culture

Dermal microvascular endothelial cells (DMECs) were isolated from human foreskin. Foreskin was digested with dispase (Corning). The epidermis was removed, and the foreskin was scraped to dislodge endothelial cells. Cells were sorted for CD31 with magnetic beads (Thermo Fisher Scientific). Endothelial cells were cultured in endothelial growth medium (EGM-2; Lonza) containing 15% fetal bovine serum (FCS; Thermo Fisher Scientific, Waltham, MA, USA) and supplements for microvascular cells (Lonza). Cells were maintained in a humidified atmosphere containing 5% CO_2 at 37 °C and passaged at 90% confluence. Prior to experiments, cells were authenticated and confirmed to be free of contamination by mycoplasma. Endothelial cells were used at passages 2–8.

2.5. Tube Formation Assay

Proangiogenic properties of PBMCsec and plasma of PBMCsec-treated whole blood were compared in a tube formation assay with human umbilical vein endothelial cells (HUVECs, passage 8) as described previously [36]. HUVECs (Lonza, Basel, Switzerland) were thawed and routinely cultured in polystyrene culture flasks (Merck Millipore, Burlington, MA, USA) containing endothelial cell basal medium-2 (EBM-2; Lonza) supplemented with endothelial cell growth medium-2 (EGM-2, Lonza) until fully confluent. The medium was changed every other day for a total of two passages. Prior to the tube formation assay, cells were maintained in EBM-2 containing 3% (*v*/*v*) heat-inactivated fetal bovine serum (Lonza) overnight and starved in basal EBM-2 without supplements for 3 h. Cells were seeded on growth factor-reduced Matrigel Matrix (Corning Inc. Life Sciences, Tewksbury, MA, USA) in µ-slides Angiogenesis (ibidi GmbH, Graefelfing, Germany) at a density of 10×10^4 cells per well and stimulated with the supernatant obtained from 3 mL of whole blood cells for 4 h. Micrographs were acquired by an inverted phase contrast microscope (CKX41 Olympus Corporation; Tokyo, Japan) equipped with a 10× objective (CAch N, 10×/0.25 PhP; Olympus) using a SC30 camera (Olympus) and cellSens Entry software (version 1.8; Olympus). Tubule formation was quantified by the Angiogenesis Analyzer plugin [37] of ImageJ (version 1.53, java 1.8.0_172) using default settings.

2.6. Protein Quantification by Enzyme-Linked Immunosorbent Assay (ELISA)

For in vitro experiments, heparinized human whole blood was drawn from male donors (age donor 1:29 years; age donor 2:29 years; age donor 3:30 years). A total of 3 mL of whole blood was centrifuged (1000× *g* for 10 min at room temperature) freshly after venipuncture or after 24 h long cultivation of whole blood in absence or presence of 12.5 units/mL PBMCsec. Plasma samples were then stored at −20 °C until further use. Protein levels of Human CXCL1, human CXCL5, human SERPINB2, human VEGF-A, and human urokinase (R&D Systems, Biotechne, Minneapolis, MN, USA) were quantified by ELISA as recommended by the manufacturer. Absorbance was measured at 450 nm by a Spark multimode microplate reader (Tecan, Männedorf, Switzerland), and analyte quantifications were determined using external standard curves.

2.7. Protease Activity Assays

To test the inhibitory effects of PBMCsec on protease activity, we performed a fluorometric enzyme activity assay (Enzcheck) using the unselective serine protease trypsin (ThermoFisher Scientific, Waltham, MA, USA) at a concentration of 0.05%. Enzyme substrate was diluted in provided assay buffer according to manufacturer's instruction. Equal amounts of trypsin were 1:2 diluted in assay buffer, control medium, or PBMCsec concentrated at 12.5 units/mL for 5 min before adding 10 µL to the prepared substrate mixture adding up to a total volume of 100 µL per well. The Urokinase Inhibitor Screening Kit (Sigma-Aldrich, St. Louis, MO, USA) was used to test the effect of the investigated plasma samples and PBMCsec on urokinase activity. In brief, 45 µL plasma sample were diluted in an equal volume of provided assay buffer. Human urokinase and substrate were added as suggested by the protocol, adding up to a total reaction volume of 100 µL per well. Samples from three donors were analyzed. For both tests, samples were then incubated at room temperature for a total of 60 min. Absorbance at 450 nm was measured by FluoStar Optima microplate reader (BMG Labtech, Ortenberg, Germany) in 15 min intervals.

2.8. Electrical Cell-Substrate Impedance Sensing (ECIS)

Electrical cell-substrate impedance sensing (ECIS, Applied Biophysics, Troy, NY, USA) was used to measure the electrical resistance of endothelial monolayers. A total of 12,000 endothelial cells were seeded on array plates (Ibidi) coated with gelatin (Sigma). After the resistance at 4000 Hz reached a stable plateau of >1000 Ω, endothelial cells were treated with indicated substances. Electrical resistance of cell monolayers was continuously monitored at 250 Hz [38].

2.9. Gel Bead-In Emulsion (GEMs) and Library Preparation

Single-cell RNA-seq was performed using the 10X Genomics Chromium Single-Cell Controller (10X Genomics, Pleasanton, CA, USA) with the Chromium Single-Cell 3′ V3 Kit following the manufacturer's instructions. After quality control, RNA sequencing was performed by the Biomedical Sequencing Core Facility of the Center for Molecular Medicine (Center for Molecular Medicine, Vienna, Austria) on an Illumina HiSeq 3000/4000 (Illumina, San Diego, CA, USA). For donor 1, we detected 2003 cells in the untreated sample and 1281 cells in the PBMCsec-treated sample, while donor 2 had 12,356 cells in the untreated sample and 10,865 cells in the PBMCsec-treated sample. Raw sequencing data were then processed with the Cell Ranger v3.0.2 software (10X Genomics, Pleasanton, CA, USA) for demultiplexing and alignment to a reference genome (GRCh38).

2.10. Data Analysis

Secondary data analysis was performed using R Studio in R (Version 4.0.4; The R Foundation, Vienna, Austria) using the R software package "Seurat" (Seurat v.4.0.0, Satija Lab, New York, NY, USA). Cells were first analyzed for their unique molecular identifiers (UMI) and mitochondrial gene counts to remove unwanted variations in the scRNA-seq data. Cells with UMI counts below 200 or above 2500 and more than 10% of mitochondrial genes were excluded from the dataset. Next, we followed the recommended standard workflow for integration of scRNA-seq datasets [39]. Data were scaled, and principal component analysis (PCA) was performed. Statistically significant principal components (PCs) were identified by visual inspection. Using the Louvain algorithm at a resolution of 0.025, we identified a total of four communities. The preselected PCs and identified clusters served for Uniform Manifold Approximation and Projection for Dimension Reduction (UMAP). After bioinformatics integration of datasets of untreated and PBMCsec-treated samples, erythrocytes were removed by excluding all cells with expression of hemoglobin subunit beta (HBB) >0.5. Clusters were then annotated on the basis of the expression of well-established cell-type-defining marker genes. We used UMAP-plots, feature plots, heat maps, volcano plots, and violin plots to visualize differences between the investigated conditions. To determine DEGs, normalized count numbers were used. We applied the FindMarkers

argument using default settings to calculate DEGs for clusters of interest between conditions with a log-foldchange threshold of 0.25 and an adjusted p-value <0.05. A $\log_2$ fold-change increase in gene expression above 1 was considered as upregulation, while a decrease below −1 was considered as downregulation. Only genes with an avgLog_2FC above 1 and below −1 were forwarded to the Metascape [40] online software package to identify significantly enriched pathways ($-\log_{10}(p\text{-value})$ >2). Additionally, the same gene sets were processed by Cytoscape plug-in ClueGO [41] to visualize significantly (p-value <0.05, kappa score: 0.4) enriched molecular functions for the investigated conditions.

2.11. Statistical Analysis

For single-cell RNA-seq, two donors were analyzed. Negative binomial regression was performed to normalize data and achieve variance stabilization. The Wilcoxon rank sum test was applied, followed by the Bonferroni post hoc test, to calculate differentially expressed genes. For in vitro experiments, at least three different donors were used. For data analysis of the tube formation assay, investigators were blinded to treatments. Data were statistically evaluated using GraphPad Prism v8.0.1 software (GraphPad Software, San Diego, CA, USA). When analyzing three or more groups, ordinary one-way ANOVA and multiple comparison post hoc tests with Dunnett's correction were calculated, and p-values <0.05 were considered statistically significant. Data are presented as the mean ± standard error of the mean (SEM).

3. Results

3.1. PBMCsec Modulates the Gene Signature of T Cells, B Cells, Granulocytes, and Monocytes

To investigate the degree to which PBMCsec alters the transcriptional landscape of immune cells in human whole blood, we conducted single-cell RNA sequencing (scRNA-seq) of whole-blood samples treated ex vivo with PBMCsec for 8 h. A methodological overview of the experimental approach employed in this study is provided in Figure 1. Bioinformatics analysis and UMAP clustering revealed four main cell populations consisting of monocytes, T-cells, B-cells, and granulocytes in all investigated conditions (Figure 2A). Identification was based on the expression of cluster marker genes including the CD14 molecule (*CD14*), CD3D molecule (CD3D), membrane spanning four domains A1 (*MS4A1*), and peptidase inhibitor 3 (*PI3*) for the respective cell types (Figure S1). Although significantly fewer cells were analyzed from donor 1, all cell types were found in both donors (Figure S2A). Treatment with PBMCsec did not result in a significant change in relative cell numbers (percentage of cells in untreated vs PBMCsec for T-cells: 75.73% vs. 74.86%; monocytes: 14.31% vs. 14.97%; B-cells: 8.89% vs. 8.09%; granulocytes 1.03% vs. 1.90%) (Figure 2B). The transcriptional heterogeneity between cell types was confirmed in a heatmap showing the average expression of cluster-defining genes of each cell type (Figure 2C). Next, we assessed changes in gene expression for all cell populations after treatment with PBMCsec. We calculated the number and distribution of significantly up- (Figure 2D) and downregulated genes (Figure 2E) for each cell type compared to the untreated control. A total number of 45 differentially expressed genes (DEG) (16 upregulated; 29 downregulated) were detected in T-cells, along with 72 in B-cells (28 upregulated, 44 downregulated) and 17 in granulocytes (two upregulated; 15 downregulated) (Figure S3A–C). Monocytes displayed the highest number of differentially expresses genes (173 upregulated; 148 downregulated), including upregulation of serpin family B member 2 (SERPINB2), epiregulin (EREG), interleukin 1 beta (IL1B), C–X–C motif chemokine ligand 1 (CXCL1), C–X–C motif chemokine ligand 3 (CXCL3), (CXCL5), and vascular endothelial growth factor A (VEGFA), and downregulation of S100 calcium-binding protein A8 (S100A8), S100 calcium-binding protein A9 (S100A9), allograft inflammatory factor 1 (AIF1), CD36 molecule (CD36), and CD163 molecule (CD163), amongst others (Figure 2F). From this, we can conclude that ex vivo stimulation of human whole blood with PBMCsec significantly changed gene expression in blood immune cells, most prominently in monocytes.

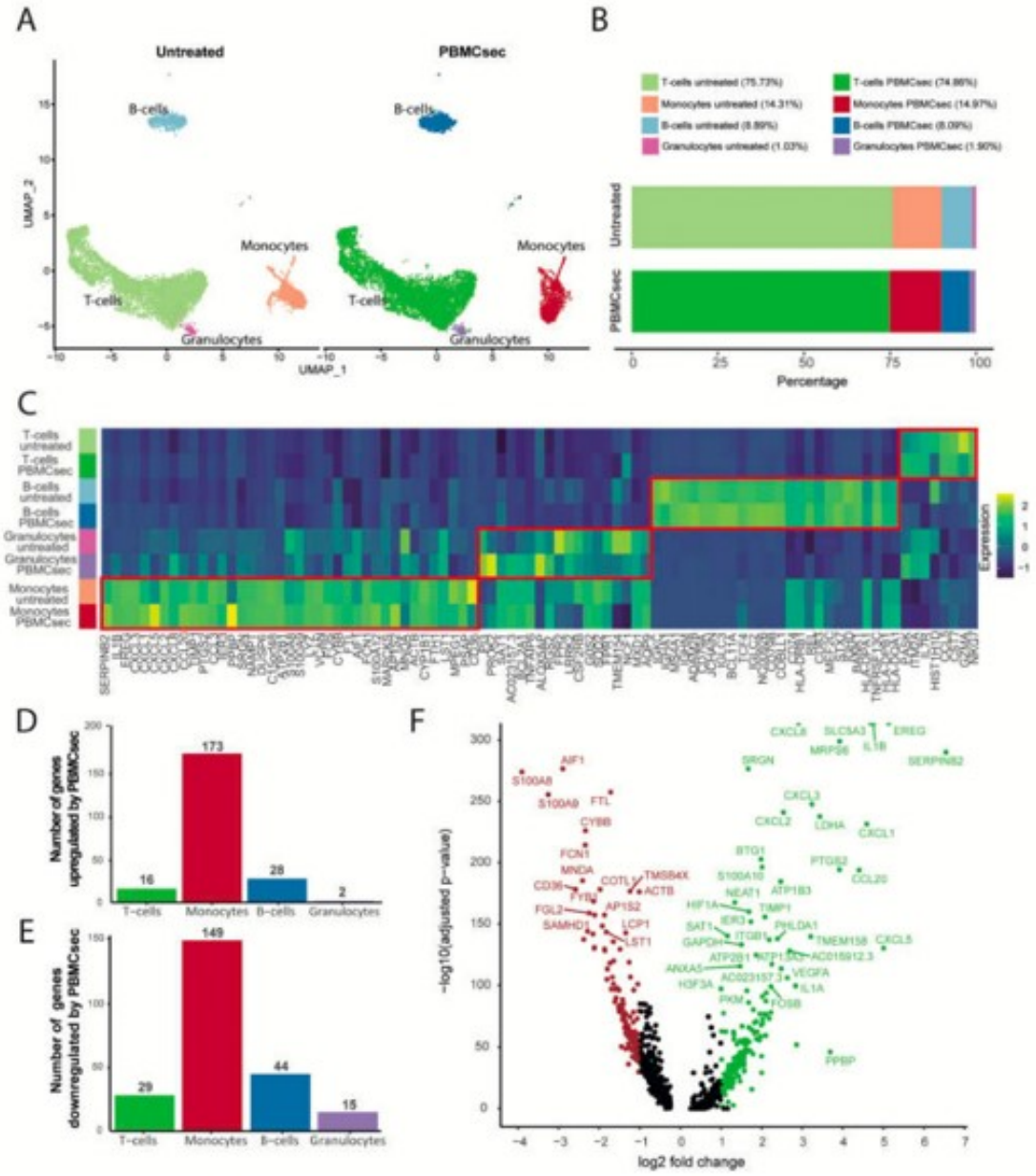

Figure 2. Ex vivo treatment of human whole blood cells alters the transcriptional profile in the monocyte subset and upregulates pathways associated with tissue regeneration. (**A**) UMAP clustering identified T-cells, monocytes, B-cells, and granulocytes in untreated and PBMCsec-treated samples with (**B**) similar cell frequency for each cluster between the investigated conditions. (**C**) Heatmap of cluster-defining marker genes of normalized gene expression showing distinct gene patterns between T-cells, monocytes, B-cells, and granulocytes. Bar plots represent the number of (**D**) up- and (**E**) downregulated genes in PBMCsec-treated samples compared to untreated control. Genes with an adjusted p-value < 0.05 and an average $\log_2$ fold change ≥ 1 or ≤ -1 were considered as DEGs. (**F**) Volcano plot showing the regulated genes in monocytes treated with PBMCsec when compared to untreated monocytes. Upregulated genes are marked in green, while downregulated genes are shown in red.

3.2. PBMCsec Induces Tissue-Regenerative Pathways in Monocytes from Human Whole Blood

Next, we identified biological pathways associated with the differentially regulated genes across the identified cell types. Biological functions such as angiogenesis and cytokine production were enriched in T- and B-cells treated with PBMCsec (Figure S3D,F), while activation of myeloid cells and responses to oxidative stress were associated with the downregulated gene sets (Figure S3E,G). No significantly regulated pathways were identified in granulocytes. The top Gene Ontology pathways associated with upregulated genes in monocytes after treatment with PBMCsec included response to lipid and to interleukin 1, along with terms strongly associated with wound healing, angiogenesis, regulation and production of cytokines, and regulation of endopeptidase activity (Figure 3A). Upregulated gene sets in monocytes treated with PBMCsec were highly comparable between the two donors (Figure S2B,C), and expression of *SERPINB2*, *VEGFA*, *CXCL1*, and *CXCL5* was significantly upregulated by PBMCsec in monocytes from both donors (Figure S2D,E), indicating a low donor variability. Major pathways associated with biological processes resulting from the downregulated gene set in monocytes treated with PBMCsec involved activation and differentiation of leukocytes, generation of reactive oxide species, and processing and presentation of antigens (Figure 3B). The complete lists of all up- and downregulated pathways (Figure S4A,B) and genes (Supplementary Files S1 and S2, respectively) are provided as Supplementary Materials. A closer look at the genes involved in the degranulation and cell activation of leukocytes revealed different members of the S100 and leukocyte immunoglobulin-like receptor (LILR) families to be downregulated in monocytes treated with PBMCsec (Figure S5A). Furthermore, we observed a downregulation of genes associated with the generation of reactive oxygen species, including the scavenger receptor CD36, the pattern recognition receptor CLEC7A, and a subunit of the NADPH oxidase complex in NCF1 (Figure S5B). We further sought to confirm our findings using ClueGO to investigate molecular functions related to these gene sets. In line with the initial analysis, we again identified strong associations with molecular functions related to cyto- and chemokine activity, signaling, and negative regulation of cysteine-type endopeptidase activity for the upregulated genes (Figure 3C), while functions related to immune receptor signaling, oxidoreductase activity, and antigen presentation were significantly associated with the set of downregulated genes (Figure 3D). From this analysis, we can conclude that monocytes were mostly affected by PBMCsec, resulting in the induction of tissue-regenerative processes, while processes associated with leukocyte activation and reactive oxygen species generation were downregulated.

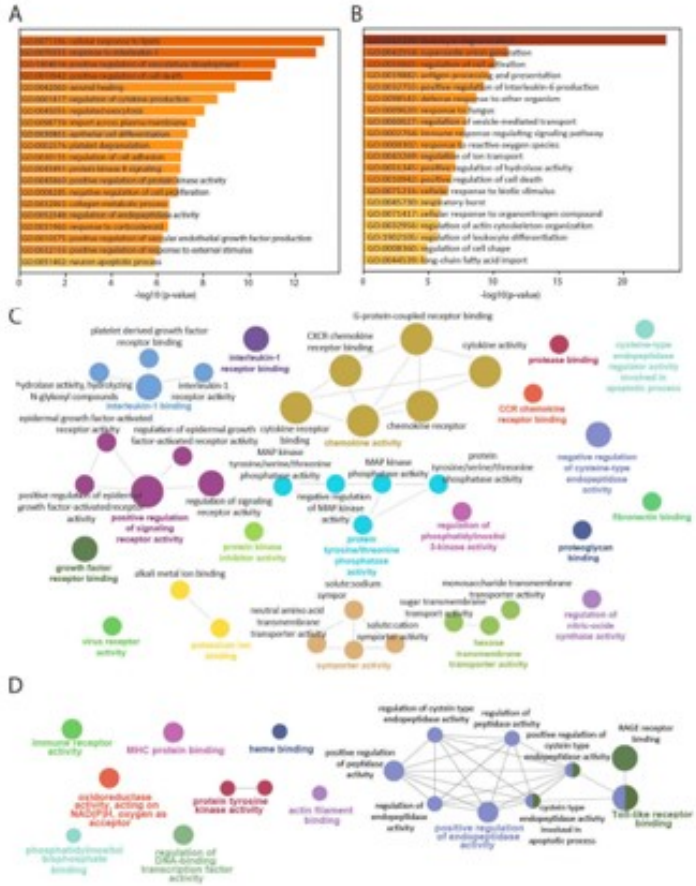

Figure 3. Tissue-regenerative pathways are upregulated in monocytes treated with PBMCsec Gene ontology pathway analysis was performed in Metascape. The top 20 biological processes affected by the upregulated (**A**) and downregulated (B) DEG in monocytes treated with PBMCsec are listed and hierarchically sorted by $-\log_{10}(p\text{-value})$ and enrichment factor (>2). (C) ClueGO visualization of molecular functions associated with upregulated and (D) downregulated genes in monocytes treated with PBMCsec.

3.3. Paracrine Factors in the Plasma of PBMCsec-Treated Whole Blood Exert Proangiogenetic Effects In Vitro

Since several cytokines and growth factors were upregulated in monocytes treated with PBMCsec (Figure 2F), we next assessed the protein levels of selected factors in plasma derived from PBMCsec-treated whole blood. PBMCsec, fresh plasma collected immediately after venipuncture, and untreated plasma after ex vivo incubation for 24 h served as controls. Gene expression of CXCL1, CXCL5, and VEGFA showed strong upregulation in monocytes after stimulation with PBMCsec (Figure 4A). This was confirmed on a protein level, when CXCL1 (2873 ± 2093 pg/mL), CXCL5 (6413 ± 1911 pg/mL), and VEGF-A (117.2 ± 13.04 pg/mL) were strongly elevated in plasma PBMCsec, while being undetectable in controls (Figure 4B). In contrast, several proinflammatory factors, such as IL-1β, TNF-α, and IFN-γ were not detectable in the plasma of PBMCsec-treated whole blood (data not shown). We next aimed to assess the proangiogenic capacity of fresh plasma, plasma of untreated whole blood, and plasma of PBMCsec-treated whole blood in an in vitro endothelial cell tube formation assay (Figure 4C). While controls showed little ability to induce tube formation, plasma of PBMCsec-treated whole blood displayed the highest proangiogenic properties as indicated by a significant increase in total segment

length, as well as in numbers of nodes and junctions (Figure 4D). In summary, we could demonstrate that the expression and secretion of proangiogenic paracrine factors were strongly increased in whole blood cells following treatment with PBMCsec.

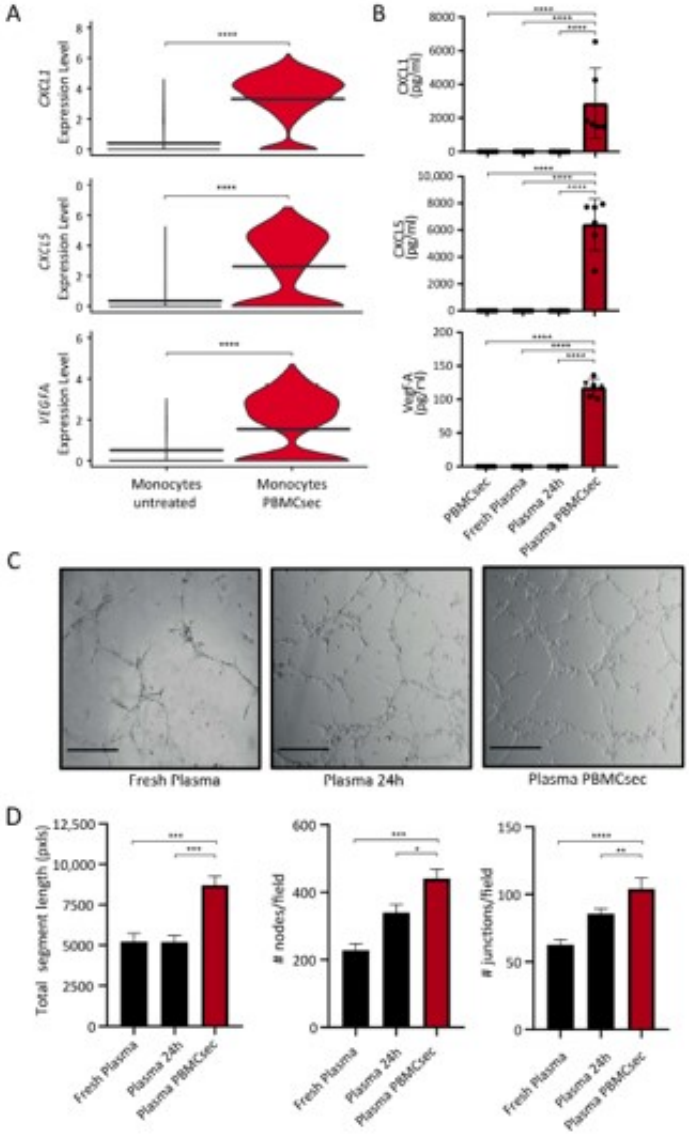

Figure 4. Paracrine factors are induced by PBMCsec in human plasma and enhance endothelial cell tube formation in vitro. (**A**) Gene expression of the proangiogenic chemokines *CXCL1* and *CXCL5*, as well as growth factor *VEGFA*, is strongly induced in monocytes treated with PBMCsec when compared to untreated monocytes (**B**) Assessment of protein levels for CXCL1, CXCL5, and VEGF-A by ELISA in plasma from PBMCsec-treated whole blood and controls (n = 3 biological replicates measured in duplicates). (**C**) Representative images of HUVEC tube formation assay in presence of fresh plasma, plasma from untreated whole blood, and plasma obtained from PBMCsec-treated whole blood after 24 h of ex vivo cultivation (scale bar 250 µm) with (**D**) analysis of number of nodes and junctions per field and total segment length (n = 3). Ordinary one-way ANOVA was performed. Dunnett's multiple comparison test was carried out to compare groups; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

3.4. PBMCsec Inhibits Protease Activity In Vitro and Induces the Selective Serine Protease SERPINB2 in Human Whole Blood Ex Vivo

Increased protease activity, as seen in acute cardiovascular or inflammatory diseases, has been shown to significantly contribute to tissue destruction, thus representing an attractive target for tissue regeneration [42,43]. Evidence from previous studies identified monocytes as a source of protease inhibitors [44]. As our pathway analysis also revealed an association of upregulated genes in monocytes with the regulation of endopeptidase activity, we next aimed to confirm this finding in functional assays in vitro. Therefore, we performed a protease activity assay to test a potential anti-proteolytic effect of PBMCsec on the nonselective serine protease trypsin. While the medium control showed a negligible inhibitory effect on protease activity (3.72 ± 0.55%), PBMCsec led to a significant inhibition of the enzymatic activity (40.51 ± 4.728%) (Figure 5A). Furthermore, SERPINB2 displayed the highest positive $\log_2$ fold-change induction (Figure 5B) of all DEGs in monocytes treated with PBMCsec in our sequencing analysis. SERPINB2 is a member of the serpin superfamily of serine proteases and encodes plasminogen activator inhibitor type II (PAI-2), which is involved in the irreversible inhibition of urokinase [45]. First, we determined the protein levels of SERPINB2 in PBMCsec, fresh plasma, plasma of untreated whole blood, and then compared them to plasma of PBMCsec-treated whole blood. In accordance with the present literature, only very low levels of SERPINB2 were detectable in the control samples. In contrast, plasma obtained from PBMCsec-treated whole blood showed significantly elevated levels of SERPINB2 (11908 ± 3530 pg/mL) (Figure 5C). As SERPINB2 mainly acts as an inhibitor for urokinase [45], we next evaluated the plasma levels of urokinase, as well as the inhibitory effect of plasma PBMCsec on its activity in vitro. Whereas high levels of urokinase were still detected (Figure 5D), urokinase activity was strongly reduced by PBMCsec-treated plasma (Figure 5E), indicating that the observed urokinase inhibitory action was a result of the presence of a urokinase inhibitor rather than a quantitative decrease in available urokinase. While in vitro urokinase activity was not affected by PBMCsec alone, addition of fresh plasma (38.45% ± 5.94%) and plasma of untreated whole blood (40.78% ± 17.07%) resulted in a considerable inhibition of urokinase activity. Interestingly, this effect was even more pronounced in the presence of plasma obtained from PBMCsec-treated whole blood (79.48 ± 2.15%) (Figure 5E). Together, these data show that soluble factors with anti-proteolytic activities were present in PBMCsec or strongly induced in white blood cells after treatment with PBMCsec.

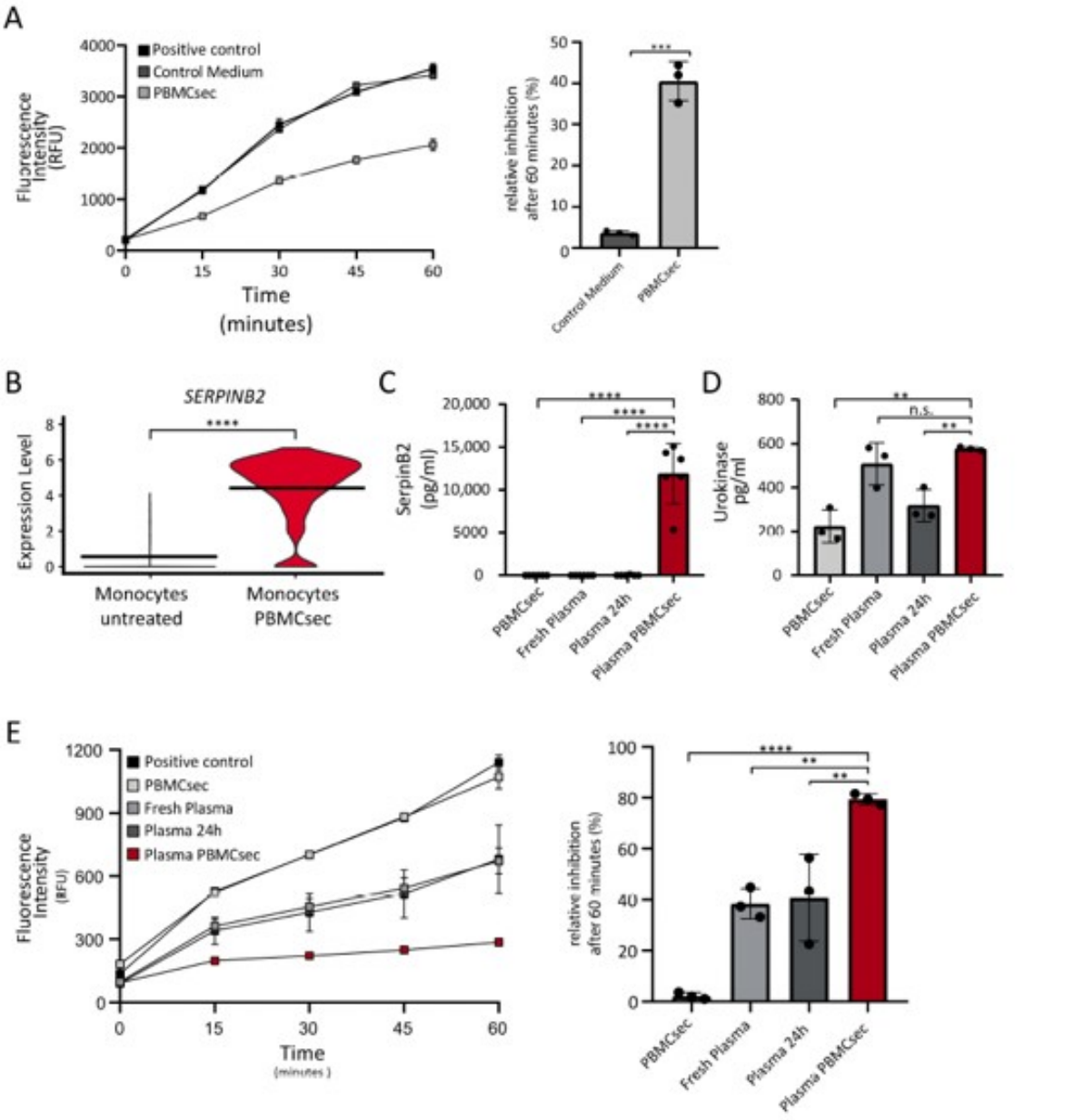

Figure 5. PBMCsec inhibits serine protease activity in vitro and increases levels of SERPINB2 in plasma of PBMCsec-treated whole blood. (**A**) Trypsin activity measured by increase in fluorescence intensity of cleaved substrate over time in the presence and absence of PBMCsec (n = 3 biological replicates). (B) Gene expression of the urokinase inhibitor *SERPINB2* in monocytes treated with PBMCsec (p-value < 0.0001) compared to untreated monocytes. (C) SerpinB2 (n = 3 biological replicates measured in duplicates) and (D) urokinase (n = 3 biological replicates) protein levels of fresh plasma, untreated plasma, and plasma of PBMCsec-treated whole blood. (E) In vitro assessment of urokinase activity of fresh plasma, plasma from untreated whole blood, and plasma obtained from PBMCsec-treated whole blood. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

3.5. PBMCsec Ameliorates Thrombin-Induced Decrease in Endothelial Barrier Function

The activity of inflammatory mediators and serine proteases has been shown to support angiogenesis, as well as increase vascular leakage, by decreasing the endothelial barrier function [38,46]. Electrical cell-substrate impedance sensing enables the continuous assessment of changes in quality and function of a cellular barrier in response to different stimuli over time [47]. Loss of barrier function in endothelial cells can result in increased extravasation of immune cells and harmful mediators into the extravasal space, further increasing tissue damage [48]. Hence, we examined whether PBMCsec affected the thrombin-induced drop in endothelial barrier function by utilizing electrical cell-substrate impedance sensing. Addition of thrombin resulted in a transient decrease in trans-endothelial resistance, reaching the lowest point 5 min after stimulation (29% ± 12% barrier function relative to untreated basal medium control) (Figure 6). While the simultaneous addition of thrombin and control medium resulted in a similar change in barrier resistance (38% ± 2%) compared to basal medium, PBMCsec inhibited the thrombin-induced decrease in endothelial resistance (84% ± 14%) (Figure 6). Therefore, PBMCsec positively influenced endothelial barrier function in vitro by attenuating thrombin-mediated changes in endothelial cells.

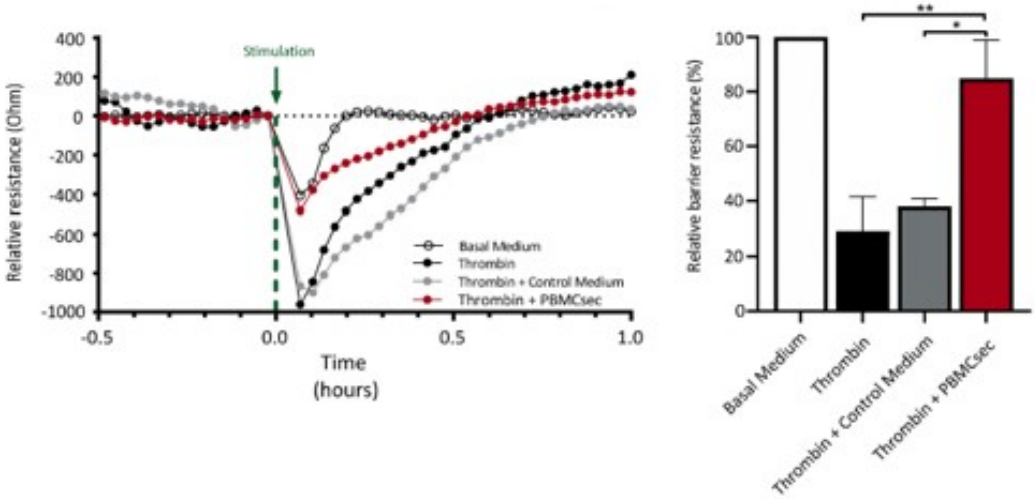

Figure 6. PBMCsec ameliorated the thrombin-induced decrease in endothelial cell barrier function. Representative evaluation of one of three independently conducted experiments. Paracellular resistance at 250 Hz was measured in DMECs challenged with 2 units/mL thrombin alone or in combination with control medium (CellGenix) or PBMCsec at 12.5 units/mL over time. The green arrow marks the time point of stimulation with the respective treatments. The bar plot shows the changes in endothelial barrier function for the investigated conditions relative to the basal medium (EBM-2) control. One-way ANOVA was performed with post hoc Dunnett's multiple comparison test to compare groups to basal medium; * $p < 0.05$, ** $p < 0.01$.

4. Discussion

The beneficial effects of PBMC-derived cell secretomes in the regeneration of damaged tissues and organs have already been well described [10,15,21,24,25]. In order to transit from these promising preclinical studies to the treatment of patients, the safety and tolerability of topical administration of autologous PBMCsec in dermal wounds was successfully assessed in a clinical phase I trial (MARSYAS I, NCT02284360) [49]. On the basis of these results, an ongoing clinical phase I/II trial has been initiated to evaluate the safety and efficacy of topically administered allogeneic PBMCsec for the treatment of diabetic foot ulcers (MARSYAS II, NCT04277598) [50]. In light of future therapeutic approaches using systemic administration of PBMCsec to regenerate inner organs, we analyzed in the present study the effects of PBMCsec on whole blood and the endothelium in more detail.

Using scRNA-seq, we identified a strong induction of tissue-regenerative pathways after treatment of human whole blood with PBMCsec ex vivo. In particular, biological processes associated with the regulation of vasculature development, wound healing, and regulation of endopeptidase activity, as well as decreased superoxide anion generation and leukocyte degranulation, all of which contribute to tissue regeneration, were significantly overrepresented in our analysis. The observed changes of the transcriptional profile of white blood cells treated with PBMCsec were most pronounced in monocytes, while all other cell types showed comparably low alterations in gene expression. Our finding is in line with previous publications where functional changes were mainly reported in monocytes after stimulation with conditioned medium from MSCs [51,52]. As monocytes are an important part of the innate immune system, a rapid response to extracellular stimuli, such as PBMCsec, was expected. Interestingly, only few granulocytes, which also represent a main constituent of innate immunity, were detected in our single-cell analysis. A low detectability of the different granulocyte populations in scRNA-seq has been reported before and might be due to their overall low RNA content and presence of RNAses [53]. This assumption is further supported by the extremely low mRNA counts in the few granulocytes detected in our analysis. Interestingly, T- and B-cells also showed only minor gene regulation after exposure to the secretome. As both cell types belong to the adaptive branch of the immune system, it is tempting to speculate that repeated exposure of lymphoid cellular subsets to PBMCsec might result in a more pronounced adaptive immune response. Furthermore, the natural process of aging may lead to various changes in immune cells from whole blood, which manifest as decreased immunological activity in older individuals. Indeed, previous studies in animal models have reported on positive age-associated effects of blood from young donors [54]. However, in light of our ongoing and future clinical studies, we here used PBMCsec produced under GMP conditions, which represents a pool of donors in the age range of 18–45 years. As pooling averages all active factors in PBMCsec, further studies will be necessary to fully address age-related effects.

Examination of the altered gene sets in monocytes revealed upregulation of several members of the chemokine (C–X–C motif) ligand (CXCL) family (*CXCL1*, *CXCL2*, *CXCL3*, *CXCL5*, and *CXCL8*), as well as different growth factors after stimulation with PBMCsec. In addition, high protein levels of CXCL1, CXCL5, and VEGF-A were detected in the plasma of PBMCsec-treated whole blood. These factors have previously been described as important constituents of PBMCsec, contributing to its tissue-regenerative actions [13,15]. Yet, we were not able to detect these proteins in PBMCsec in our assays. This discrepancy could be explained by the lower concentration of PBMCsec that we used in our ex vivo study. The lower concentration of PBMCsec had to be used for the stimulation of PBMCs, and endothelial cells as 1:2 dilutions with fresh medium were necessary for cell viability. Nevertheless, all these factors were strongly induced in whole blood and abundantly present in the plasma derived from PBMCsec-treated whole blood, indicating a significant amplification of the production of tissue regenerative factors. In addition, our transcriptional analysis revealed a downregulation of genes involved in the generation of superoxide anion species with an inhibitory effect on the formation of reactive oxygen species in PBMCsec-treated monocytes. Expression of the scavenger receptor CD36 on monocytes treated with PBMCsec was amongst the most strongly downregulated genes. In monocytes and macrophages, activation of this receptor promotes increased production of reactive oxygen species, which in turn reinforced damage to the vasculature [55]. Furthermore, CD36 is known to modulate the uptake of oxidized lipid species by macrophages, leading to functional changes in these cells. As they accumulate lipids, these so-called foam cells then promote proliferation and inflammation of endothelial and smooth muscle cells, which contributes to the formation of atherosclerotic plaques [56]. Given that reactive oxygen species increase after re-establishment of perfusion and aggravate tissue damage in a series of cardiovascular pathologies, our data offer a potential mechanism via which PBMCsec might directly and/or indirectly reduce tissue damage after ischemic conditions.

Future studies will elucidate the influence of PBMCsec on the generation and amelioration of reactive oxygen species.

Various biological active components have already been identified, contributing to the diverse effects of PBMCsec, including cytokines, growth factors, lipids, and exosomes. In particular, lipids present in PBMCsec have been shown to strongly impact immune cell functions. Laggner et al., recently demonstrated that PBMCsec-derived lipids interfere with dendritic cell differentiation and mast cell and basophil granulocyte degranulation, thereby improving skin inflammation and allergic symptoms, respectively [19,20]. This special composition of biologically active cytokines, growth factors, lipids, and exosomes, amongst other yet unidentified substances, makes PBMCsec a highly effective compound against tissue damage and inflammation. Previous work from our group demonstrated that paracrine factors released from γ-irradiated PBMCs positively influence tissue regeneration by affecting endothelial cell survival and sprouting [12]. In addition, we here showed that PBMCsec was also able to inhibit thrombin-induced disruption of the endothelial barrier in dermal microvascular endothelial cells. This effect might counteract increased leakage of blood vessels, thereby preventing insufficient perfusion of the vital part of the injured organ and amplification of the damage. We also observed an inhibitory effect of the plasma of PBMCsec-treated whole blood on the serine protease urokinase, which was not detected in PBMCsec alone. Thus, de novo synthesis of the urokinase inhibitor SERPINB2 could mediate this effect. Serine protease inhibitors are widely discussed as promising therapeutic approaches for several disease entities. Recently, Vorstandlechner et al. identified dipeptidyl-peptidase 4 (DPP4) and urokinase (PLAU) as important drivers of skin fibrosis, and targeted inhibition of both molecules was shown to reduce myo-fibroblast formation and improve scar quality [57]. Implications for therapeutic targeting of serine proteases are also evident for myocardial infarction [58,59]. While early re-vascularization of occluded blood vessels still remains the gold-standard intervention to maximize rescue of functional tissue, increasing emphasis is being put on the reduction of late onset events caused by accumulation of proinflammatory stimuli, proteases, and other detrimental factors, secondary to reperfusion injury [32]. Mauro and colleagues reported reduced infarction sizes following treatment with alpha-1-antitrypsin, in addition to revascularization, in a murine model of myocardial infarction [60]. This was further reinforced by a report by Hooshdaran et al., where inhibition of the serine proteases cathepsin G and chymase was shown to reduce adverse cardiac remodeling, myocyte apoptosis, and fibrosis in a murine model of myocardial ischemia reperfusion injury [61]. In addition, a recent publication by Sen and colleagues reported a central role for SerpinB2 in the coordinated resolution and repair of damaged tissue after ischemia/reperfusion in a murine kidney injury model [62]. These data suggest that, in addition to the protective action of serine protease inhibitors investigated in our study, they might also show valuable tissue-regenerative and antifibrotic properties. Further studies are needed to fully decipher the contribution of protease inhibitors already present in PBMCsec, as well as de novo induced inhibitors such as SerpinB2, and their respective roles in the inhibition of tissue-damage or the restoration of damaged tissue and organs.

Paracrine signaling for the prevention of a proinflammatory response and cell death, as well as induction of angiogenesis, is essential to restore damaged tissues and organs [52,63]. We previously showed that PBMCsec was able to positively modulate all of these events [12,15,64], and administration of a single dose of PBMCsec was sufficient to almost completely inhibit heart damage in a porcine model of experimental myocardial infarction [15]. However, given that pharmacodynamic investigations revealed that several main components of PBMCsec were only traceable for minutes to a maximum of 5 h in the blood of rats and dogs after intravenous application of human PBMCsec, this finding was rather unexpected [65]. Whether human PBMCsec induces a comparable release of pro-regenerative factors in these animal models has not been investigated so far. Our new data provide a reasonable explanation for this observation and suggest that PBMCsec induces the production of a new secretome in human white blood cells with additional

regenerative properties. This is in line with our findings of increased endothelial cell sprouting after treatment of HUVECs with plasma of PBMCsec-treated whole blood. Cell types other than PBMCs, which could also contribute to the regenerative effects, can be found in whole blood. Thrombocytes are known for their proangiogenic properties, which are mainly due to the release of VEGF upon thrombocyte activation [66]. Interestingly, previous work from our group identified an inhibitory effect of PBMCsec on platelet activation and aggregation [18], suggesting that PBMCsec does not promote the release of proangiogenic mediators from thrombocytes. In contrast, an increased activity was observed in untreated plasma after cultivating whole blood for 24 h, as compared to fresh plasma, which might be a result of thrombocyte activation in the absence of PBMCsec. Importantly, our results indicate that the pharmacodynamic effect of a single application of PBMCsec can be significantly prolonged by continuous stimulation, comparable to the behavior of a damped wave. Therefore, this induced secretome, produced over an extended period of time, might multiply the pro-regenerative properties of PBMCsec by combining them with those of the newly induced factors. However, further studies in a human setting will be necessary to fully explore the tissue-regenerative potential of the newly formed secretome and to examine for how long this effect can be maintained.

In summary, our scRNA-seq analysis identified key mechanisms, potentially contributing to tissue regeneration, which might occur after systemic application of a cell secretome derived from irradiated PBMCs. PBMCsec displays a broad spectrum of mechanistic modes of actions that greatly complement each other and, therefore, positively contribute to tissue regeneration in a wide range of pathological settings. In addition, our data suggest that the effects in vivo are not restricted to direct actions of PBMCsec but also arise from further stimulation of circulating monocytes in the blood. Further human clinical studies in the future will clarify the underlying mechanisms and the therapeutic benefit of systemic treatment of damaged organs with PBMC-derived secretomes.

5. Patents

The Medical University of Vienna has claimed financial interest. H.J.A. holds patents related to this work (WO 2010/079086 A1; WO 2010/070105 A1; EP 3502692; European Patent Office application #19165340.1).

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/pharmaceutics14081600/s1: Figure S1. Identification of cell clusters based on expression of cluster-defining marker genes for T-cells, B- cells, monocytes, and granulocytes; Figure S2. Donor comparability of detected cell types and identification of pathways associated with upregulated genes in monocytes treated with PBMCsec; Figure S3. Transcriptional changes in T-cells, B-cells, and granulocytes and their predicted regulation of biological processes; Figure S4. Biological processes affected by up- and downregulated genes in monocytes treated with PBMCsec; Figure S5. Downregulated genes in monocytes treated with PBMCsec associated with leukocyte degranulation and generation of reactive oxygen species; Supplementary sheet 1. Pathways associated with significantly upregulated genes in monocytes treated with PBMCsec; Supplementary sheet 2. Pathways associated with significantly downregulated genes in monocytes treated with PBMCsec.

Author Contributions: M.M., H.J.A. and D.C. conceptualized and planned the experiments; H.J.A. and M.M. acquired funding; D.C., M.D. and K.S. performed the experiments; D.C., M.D., K.S., M.L., K.K., D.B., H.J.A. and M.M. participated in data interpretation; D.C., M.L. and M.M. drafted the manuscript. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: This study was conducted in accordance with the Declaration of Helsinki and local regulations. Use of primary HUVECs, primary DMECs, and blood samples was

approved by the Institutional Review Board of the Medical University of Vienna (Ethics committee votes: 1280/2015, 1539/2017, and 1621/2020). All donors provided written informed consent.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: ScRNA-seq data are available upon request.

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2.1.1 Interlude

ATG has successfully been utilized in transplantation medicine for treatment of T-cell-mediated rejections as well as for conditioning of transplant recipients at high risk of graft rejection. The effects associated with ATG are generally attributed to its T-cell depleting properties. It is well known that ATG induces programmed cell death via various distinct pathways. Similarly to PBMCsec, ATG-mediated cell death leads to the release of large quantities of paracrine factors which in turn influence the immunological functions in distinct subsets of the immune system. The influence of these factors on immune cell modulation remains elusive. Therefore, we examined the influence of ATG on immune cells from human whole blood with particular emphasis on released paracrine factor and how these shape immunological responses and cell-cell interactions of lymphoid and myeloid subsets.

2.2 Second Publication

cells

MDPI

Article

Antithymocyte Globulin Inhibits CD8⁺ T Cell Effector Functions via the Paracrine Induction of PDL-1 on Monocytes

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Abstract: Background: Antithymocyte globulins (ATG) are T cell-depleting antibodies used in solid organ transplantation for induction therapy in sensitized patients with a high risk of graft rejection. Previously described effects besides the depletion of T cells have suggested additional modes of action and identified further cellular targets. Methods: We examined the transcriptional changes arising in immune cells from human blood after ex vivo stimulation with ATG at the single-cell level to uncover additional mechanisms by which ATG regulates T cell activity and effector functions. Findings: Analysis of the paracrine factors present in the plasma of ATG-treated whole blood revealed high levels of chemokines and cytokines, including interferon-γ (IFN-γ). Furthermore, we identified an increase in the surface expression of the programmed death ligand 1 (PDL-1) on monocytes mediated by the released paracrine factors. In addition, we showed that this induction is dependent on the activation of JAK/STAT signaling via the binding of IFN-γ to interferon-γ receptor 1 (IFN-γR1). Lastly, we demonstrated that the modulation of the immune regulatory axis of programmed cell death protein 1 (PD1) on activated CD8⁺ T cells with PDL-1 found on monocytes mediated by ATG potently inhibits effector functions including the proliferation and granzyme B release of activated T cells. Interpretation: Together, our findings represent a novel mode of action by which ATG exerts its immunosuppressive effects.

Keywords: antithymocyte globulin; inhibitory co-stimulation; single-cell RNA sequencing; T cell activation

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1. Research in Context

For individuals with a high risk of graft rejection, induction therapy with cell-depleting antibodies in addition to standard immunosuppressive regimens has been shown to reduce the risk of acute rejection in solid organ transplantation. Antithymocyte globulin (ATG) has previously been shown to deplete T lymphocytes and thus improve graft survival after transplantation. However, recent data suggest additional modes of action which are not restricted to T lymphocytes but also affect myeloid cell populations such as monocytes. The exact mechanisms have not been identified yet.

2. Added Value of This Study

We were able to demonstrate the induction of the immunosuppressive receptor–ligand PDL-1 on monocytes following ex vivo stimulation of human whole blood and purified PBMCs with ATG. Furthermore, we show that the induction is mediated by paracrine actions and activation of the JAK/STAT signaling pathway in monocytes. In addition, PDL 1⁺ monocytes reduce the proliferative rate and secretion of granzyme B of activated CD8⁺ T lymphocytes in vitro.

3. Implications of All the Available Evidence

The identification of this novel immunosuppressive mechanism extends the previous understanding of ATG and its mode of action and offers a novel target for investigation in patient-relevant settings.

4. Introduction

The induction and maintenance of immunosuppressive therapy has strongly contributed to reducing graft rejections after solid organ transplantation [1]. In patients with high immunological risk of acute graft rejection, antithymocyte globulin (ATG) is recommended for induction therapy [2,3]. ATG is a mixture of purified polyclonal γ-globulins obtained from the sera of horses or rabbits inoculated with cells from the human thymus [4]. These antibodies bind to a vast array of antigens found on the cell surface of human T lymphocytes as well as other immune cells [5]. ATG depletes T lymphocytes via classical complement-dependent cell cytotoxicity (CDCC), antibody-dependent cell cytotoxicity (ADCC), opsonization of reactive T cells and the induction of the activation-induced cell death pathway (AICD) [6–9]. Together, these mechanisms are responsible for the rapid and profound lymphopenia observed in patients treated with ATG. However, during or shortly after completion of induction therapy, the number of circulating T cells in the blood gradually increases and reverts to normal values [10]. Interestingly, the recurring T lymphocytes display impaired proliferative capacity even after the termination of ATG therapy [11]. Therefore, additional immunosuppressive mechanisms besides the direct T cell-depleting effects are apparent, including the masking of antigens, increased clearance of T lymphocytes via the reticuloendothelial system and expansion of regulatory T cells (T_{regs}) [11–14]. Moreover, ATG-mediated effects on the immune system are not restricted to direct actions on T lymphocytes, as previous studies demonstrated an induction of B lymphocyte apoptosis [15], immunomodulation of natural killer cells [16] and inhibition of in vitro monocyte-derived dendritic cell maturation [17,18]. However, the underlying mechanisms responsible for the latter effect are not fully understood.

Immunological responses in T cells require the engagement of the T cell receptor (TCR) together with a presented antigen and a second co-stimulatory signal [19]. Depending on the co-stimulatory signal, T cell effector functions are either promoted or dampened [20]. Continuous stimulation of the TCR due to ongoing antigen presentation, as seen after solid organ transplantation, has been shown to upregulate the inhibitory co-receptor programmed cell death protein 1 (PD-1) on the surface of activated T cells [21]. Ligation of this receptor by its ligand programmed death receptor ligand 1 (PDL-1) transduces an inhibitory signal in activated T lymphocytes and decreases their effector functions, including proliferation and cytokine release [22]. Several studies have already reported on the importance of the PD1:PDL-1 axis for the maintenance of autoimmunity and peripheral tolerance after solid organ transplantation [23–25]. However, the involvement of the PD1:PDL-1 system in ATG-induced immunosuppression has not been described so far.

In this study, we examined the transcriptional changes in ATG-treated circulating immune cells from human blood at the single-cell level in order to elucidate novel mechanisms by which ATG exerts its immunosuppressive effects.

5. Materials and Methods

5.1. Study Design

The aim of this study was to investigate the changes induced by the ex vivo treatment of human whole blood with ATG and uncover potential novel mechanisms by which ATG regulates T lymphocyte functions. To this end, we used single-cell RNA sequencing to identify transcriptional alterations set in motion by ATG in different immunological subsets of lymphoid and myeloid white blood cells. For scRNA-seq, two donors were sampled per experimental group.

To verify our bioinformatics data, we performed a series of cell culture- and flow cytometry-based assays. For the in vitro experiments involving purified PBMCs and

purified monocytes, the number of individual replicates as well as statistically significant differences are shown in the figure legends, and we included at least three donors per experiment. We examined the effects of ATG-mediated paracrine factors on purified monocytes and identified a crucial role of IFN-γ in the induction of the immune regulatory co-receptor PDL-1. We also delineated the functional contributions of PDL-1$^+$ monocytes on activated CD8$^+$ T cells in vitro.

5.2. Ethics Statement

This study was conducted in accordance with the Declaration of Helsinki and local regulations. Blood samples were obtained from healthy volunteers who had given their consent to donate prior to participation. The Institutional Review Board of the Medical University of Vienna approved this study (Ethics Committee votes: 1539/2017). All donors provided written informed consent.

5.3. Preparation of Single-Cell Suspension of Human Whole Blood

For scRNA-seq, heparinized human whole blood was drawn from two age-matched male donors. A total of 3 mL of whole blood was either treated with 100 µg/mL ATG (Grafalon; Neovii Biotech GmbH, Gräfelfing, Germany), an equivalent dose of polyclonal rabbit isotype control (ab37415; Abcam, Cambridge, UK) or left untreated. The used dosage corresponded to the blood levels detected in patients undergoing ATG induction [26]. Samples were incubated at 37 °C for 8 h. Next, red blood cells were removed by red blood cell lysis buffer (Abcam). Cells were then washed twice with PBS containing 0.04% bovine serum albumin (BSA; Gibco) and sequentially passed through 100 and 40 µm cell strainers. The LUNA-FL Dual Fluorescence Cell Counter (BioCat, Heidelberg, Germany) and the Acridine Orange/Propidium Iodide Cell Viability Kit (Logos Biosystems, Gyeonggi-do, South Korea) were used to adjust the cell count to 1×10^6 cells/mL with a viability above 90%.

5.4. Gel Bead-in Emulsions (GEMs) and Library Preparation

Single-cell RNA-seq was performed using the 10X Genomics Chromium Single Cell Controller (10X Genomics, Pleasanton, CA, USA) with the Chromium Single Cell 3′ V3 Kit following the manufacturer's instructions. After quality control, sequencing was performed by the Biomedical Sequencing Core Facility of the Center for Molecular Medicine (Center for Molecular Medicine, Vienna, Austria) on an Illumina HiSeq 3000/4000 (Illumina, San Diego, CA, USA). For donor 1, we detected 2094 cells in total, while for donor 2, altogether 18,257 cells were captured. Raw sequencing data were then processed with the Cell Ranger v3.0.2 software (10X Genomics, Pleasanton, CA, USA) for demultiplexing and alignment to a reference genome (GRCh38).

5.5. Single-Cell RNA Sequencing Data Analysis

Secondary data analysis was performed using R Studio in R (The R Foundation, Vienna, Austria) using the R software package "Seurat" (Seurat v.4.0.0; Satija Lab, New York, NY, USA). Cells were first analyzed for their unique molecular identifiers (UMI) and mitochondrial gene counts to remove unwanted variations in the scRNA-seq data. Cells with feature RNA counts below 100 or above 2500 and more than 10% of mitochondrial genes were excluded from the dataset. Next, we followed the recommended standard workflow for the integration of scRNA-seq datasets [27]. Data were scaled and principal component analysis (PCA) was performed. Statistically significant principal components (PCs) were identified by visual inspection using an elbow plot. Calculation of the Louvain algorithm at a resolution of 0.2 iterations identified a total of 11 communities. The preselected PCs and identified clusters served as the uniform manifold approximation and projection for dimension reduction (UMAP). After the bioinformatics integration of the datasets of untreated, isotype-treated and ATG-treated samples, erythrocytes were removed by excluding all cells with the expression of hemoglobin subunit beta (HBB) > 0.5. Annotation of cell types to the calculated clusters was based on the expression of cell type-specific marker genes that

were determined with the "FindAllMarkers" argument in Seurat. We used UMAP plots, dot plots, feature plots, volcano plots and violin plots to visualize differences between the investigated conditions. Normalized count numbers were used to determine DEGs. We applied the "FindMarkers" argument using the default settings to calculate DEGs for clusters of interest between conditions. A $\log_2$-fold change increase in gene expression above 1 was considered as upregulation, while a decrease below −1 was considered as downregulation. The sets of DEGs were imputed into the Cytoscape [28] plug-in ClueGO [29] to visualize significantly (p-value < 0.05, kappa score: 0.4) overrepresented gene ontologies related to immune system processes for the investigated conditions.

5.6. Gene Set Enrichment Analysis (GSEA)

GSEA of gene ontologies was conducted in R using the Bioconductor package "clusterprofiler" [30]. A list of DEGs was calculated comparing every ATG-treated cell type with its matched isotype-treated control. This list was then sorted in descending order for each cell type. For gene annotation, the "org.Hs.eg.db." package was loaded and executed in R. Non-annotated genes were omitted from further analysis. Using the "gseGO" command, we calculated significantly (p-value < 0.05) enriched gene ontologies for biological processes (GO: BP) in the ATG-treated cell types. The default settings for minimal and maximal gene set size as well as the number of permutations were maintained for initial analysis. To adjust the calculated p-values and minimize the false discovery rate, we performed a Benjamini–Hochberg correction. Results were visualized by GSEA plots embedded in the "ggplot2" package.

5.7. CellChat Analysis for Ligand–Receptor Interactions

To infer cellular communications between the identified cell types in our scRNA-seq analysis, we used the R package "CellChat" [31]. Firstly, we converted our initial Seurat object into a CellChat object. Next, the CellChat database of receptor and ligand pairs was implemented into the new object. Then, we performed an overrepresentation analysis for genes and possible interactions within the CellChat database. The p-value threshold for a significant ligand–receptor interaction was set to <0.05. Cell–cell interactions are displayed as chord diagrams.

5.8. Isolation of PBMCs and Cell Purification

For in vitro assays, PBMCs were isolated using density gradient centrifugation with Ficoll–Paque PLUS (GE Healthcare Bio-Sciences AB, Sweden, Uppsala). Heparinized blood was diluted with phosphate-buffered saline (PBS; Gibco by Life Technologies, Carlsbad, CA, USA) and carefully layered over Ficoll–Paque PLUS. After centrifugation (800× g, 15 min, room temperature, with slow acceleration and deceleration), buffy coats containing PBMCs were enriched at the plasma–Ficoll interface. For the purification of $CD14^+$ monocytes, $CD2^+$ cells and $CD8^+$ T cells, we performed magnetic bead separation using CD14, CD2 and CD8 magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) to enrich cells over the QuadroMACS™ Separator (Miltenyi) according to the manufacturer's protocol. The purity of isolated cells was confirmed by flow cytometry using antibodies against CD14-PE (BioLegend, San Diego, CA, USA; clone: HCD14) and CD8-PeCy5 (BioLegend; clone: SK1) and ranged from 95 to 99%. A complete list of all antibodies used in this study is provided in Supplementary Table S1. Acquired cells were counted, diluted to a concentration of 1×10^6 cells/mL and cultured in RMPI 1640 supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Waltham, MA, USA) and 1% Pen-Strep (Thermo Fisher Scientific) unless otherwise stated. After 24 h of stimulation with ATG, the PDL-1-FITC (BioLegend, clone: MIH2) antibody was used to assess surface expression on the immune cells.

5.9. Proteome Profiler and Enzyme-Linked Immunosorbent Assay

Whole blood samples and isolated PBMCs were treated with ATG (100 µg/mL) or an isotype control (100 µL/mL) and cultured in standard cell culture conditions for 24 h. Next, samples were collected and centrifuged at 1000× *g* for 10 min to obtain cell-free plasma and conditioned media of PBMCs. Supernatants were passed through a 0.22 µm filter before storage at −20 °C until further use. To assess the cytokine profile of the different conditions, plasma- and PBMC-conditioned media from three donors were pooled separately and subjected to the Proteome Profiler Assay Human XL Cytokine Array Kit (R&D Systems, Minneapolis, MN, USA). The assay was performed according to the manufacturer's instructions. Signals were developed using the Gel Doc XR+ device (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and dot densities for each cytokine duplicate were calculated using the volume tool in ImageLab 6.0.1 (Bio-Rad Laboratories, Inc.). For visualization, the bar plot option of GraphPad Prism was used (v.8.0.1; GraphPad Software, La Jolla, CA, USA) to display differences detected in the ATG-treated plasma and PBMC supernatant as the fold change relative to the controls. In addition, we measured IFN-γ in supernatants of ATG-treated whole blood, purified PBMCs and purified $CD8^+$ T cells as well as human granzyme B in co-cultures via ELISA (Bio-techne, R&D, Minneapolis, MN, USA).

5.10. Flow Cytometry

Flow cytometry was performed on a BD FACSCanto II flow cytometer and data were analyzed using FlowJo (10.6.2) software (Tree Star, Ashland, OR, USA). A list of the antibodies used for the detection of cellular epitopes of interest is provided in Supplementary Table S1.

5.11. INF-γR1 and IFN-γR2 Blockade

Isolated monocytes were cultured at 1×10^6 cell/mL in cell culture medium in the presence of the anti-IFN-γR1 antibody (Bio-techne) for 2 h. Monocytes without added antibody served as controls. Next, the conditioned medium obtained from untreated, isotype-treated and ATG-treated PBMCs was added at a ratio of 1:10. Cells were cultured for a total of 24 h before the flow cytometric assessment of PDL-1 surface expression on monocytes.

5.12. Inhibition of JAK/STAT Signaling in Purified Monocytes

Purified monocytes were treated with 10 µm of the JAK/STAT inhibitor ruxolitinib (MedChemExpress, Princeton, NJ, USA) diluted in DMSO (dimethyl sulfoxide; Merck, Darmstadt, Germany) for 1 h before the addition of conditioned media of untreated, isotype-treated and ATG-treated PBMCs at 1:10. After two hours, one replicate of monocytes was lysed in 1× Laemmli buffer (Bio-Rad Laboratories, Inc.) supplemented with protease and phosphatase inhibitor for the assessment of total Stat 1 (Cell Signaling Technology, Danvers, MA, USA) as well as phosphorylated Stat 1 (Cell Signaling Technology) by Western blot. The remaining cells were cultured for up to 24 h before determining the surface expression of PDL-1 via flow cytometry.

5.13. Western Blotting

Purified monocytes were lysed in 1× Laemmli Buffer (Bio-Rad Laboratories, Inc.) supplemented with protease and phosphatase inhibitor and loaded on 4–15% SDS–PAGE gels (Bio-Rad Laboratories, Inc.). Proteins were transferred on a nitrocellulose membrane (Bio-Rad Laboratories, Inc.), blocked in non-fat milk with 0.1% Tween 20 (Sigma-Aldrich) for 1 h, and incubated with antibodies as indicated in Supplementary Table S1 at 4 °C overnight. After washing, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Signals were developed with SuperSignal West Dura substrate (Thermo Fisher Scientific) and imaged with a Gel Doc XR+ device (Bio-Rad Laboratories, Inc.). Quantification analysis was performed using the Volume tool in ImageLab 6.0.1 (Bio-Rad Laboratories, Inc.).

5.14. Co-Culture of Activated CD8+ T Cells with Monocytes

CD8+ T cells were isolated from whole blood using CD8+ magnetic beads (Miltenyi) and a QuadroMACS™ separator (Miltenyi). In total, 5×10^6 cells/mL were stained with the CellTrace™ violet proliferation kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Next, anti-CD3 and anti-CD28 T cell activation beads were added to the isolated T cells before placing them in a round-bottom 96-well plate. A total of 5×10^5 T cells was pipetted into each well. After 24 h of activation, the same number of autologous monocytes was added to the T lymphocytes. Prior to addition, monocytes were treated with conditioned media of untreated PBMCs or ATG-treated PBMCs for 24 h and subsequently washed twice. In addition, we incubated PDL-1+ monocytes for 1 h with durvalumab (Imfinzi; MedImmune, AstraZeneca, Cambridge, UK), a monoclonal antibody directed against PDL-1, to assess its contribution to the inhibition of CD8+ T cell proliferation. Experiments were performed with four independent donors. After 4 days of co-culture, cells were collected and stained for CD8-PECy5 (BioLegend) and assessed for cell proliferation with flow cytometry. Conditioned media of co-cultured cells were collected and stored at −20 °C until further use.

5.15. Statistical Analysis

For single-cell RNA-seq, two donors were analyzed. Negative binomial regression was performed to normalize the data and achieve variance stabilization. The Wilcoxon rank sum test was followed by the Benjamini–Hochberg post hoc test to calculate differentially expressed genes. For in vitro experiments, at least three different donors were used. Data were statistically evaluated using GraphPad Prism v8.0.1 software (GraphPad Software, San Diego, CA, USA). When analyzing three or more groups, ordinary one-way ANOVA and multiple comparison post hoc tests with Dunnett's correction were performed, with p-values < 0.05 considered as statistically significant. Data are presented as the mean ± standard error of the mean (SEM).

6. Results

6.1. ATG Alters the Transcriptional Profile of Lymphoid and Myeloid Immune Cells from Human Whole Blood

To elucidate the effects of ATG beyond T lymphocyte depletion, as well as its influences on myeloid immune cells, we investigated the transcriptional changes resulting from treatment with ATG. Single-cell RNA sequencing (scRNA-seq) of untreated, isotype- and ATG-treated (ATG) human whole blood revealed 11 distinct cell clusters in each of the investigated conditions (Figure 1A). Cell types were assigned to clusters based on the expression of established marker genes (Figure S1A). We identified different subsets of *CD4*+ T cells (naive *CD4*+ T cells, effector *CD4*+ T cells, Tregs), *CD8*+ T cells (naive *CD8*+ T cells, effector *CD8*+ T cells 1, effector *CD8*+ T cells 2) as well as NK/T cells, B cells, monocytes, *FCGR3A*+ monocytes and dendritic cells. The relative cluster frequencies across all conditions were highly similar, with the exception of naive *CD4*+ T cells, which accounted for 41.81% of all captured cells in ATG as opposed to 26.14% and 28.18% in untreated and isotype-treated cells, respectively (Figure 1A). ATG altered gene expression in all cell types relative to the isotype-treated control (Figure S1B). In the lymphoid subsets, we were particularly interested in the influences of ATG on cell types with cytolytic effector functions. Effector *CD8*+ T cells 1 (104 upregulated, 64 downregulated), effector *CD8*+ T cells 2 (39 upregulated, 43 downregulated) and NK/T cells (116 upregulated, 105 downregulated) showed considerable changes after treatment with ATG. As they hold high functional resemblance, we next determined the overlapping transcriptional regulations that were induced by ATG in these cell types. Between them, 31 genes were commonly upregulated by ATG (Figure S1C), while 29 were downregulated (Figure S1D). Amongst the upregulated genes, we identified several members of the chemokine gene family (*CCL2*, *CCL3*, *CCL3L1*, *CCL4*, *CCL8*, *CXCL8*, *XCL1* and *XCL2*), the TNF receptor superfamily (*TNFRSF4* and *TNFRSF9*) and *IFNG* (Figure S1C). Interestingly, the myeloid

cell types captured in our analysis were also strongly affected by treatment with ATG, resulting in an upregulation of 173, 119 and 76 genes, whereas 97, 77 and 40 were downregulated (Figure S1B) in ATG-treated monocytes, $FCGR3A^+$ monocytes and dendritic cells, respectively. Amongst the 33 genes that were commonly upregulated by ATG in these myeloid cell types, we identified the IFN-γ response chemokines *CXCL9*, *CXCL10*, *CXCL11* as well as *CD274* (Figure S1E), which encodes for programmed death-ligand 1 (PDL-1) [32]. Monocytes were the most abundant myeloid cell population captured in our analysis and revealed the most transcriptional changes (Figure 1A and Figure S1B). Significantly regulated genes with the highest log_2-fold changes in monocytes treated with ATG compared to those treated with the isotype control are shown in Figure 1B, while volcano plots highlighting the regulations in the other cell types are provided as supplementary information (Figure S2). An overrepresentation analysis for immune system processes affected by genes upregulated in monocytes of ATG-treated whole blood revealed significant associations with cellular responses to interferon-γ, immune cell chemotaxis, antibody-dependent cellular cytotoxicity and the regulation of $CD8^+$ T-cell activation (Figure 1C). Genes downregulated in ATG-treated monocytes were associated with immune system processes involved in macrophage activation and migration, amongst others (Figure 1D). In line with the transcriptional alterations, the analysis of the plasma of ATG-treated whole blood showed an increased protein release of several cytokines and chemokines. In total, we detected 12 factors that were significantly increased in the plasma of ATG-treated whole blood compared to isotype-treated controls (Figure 1E), including members of the CCL or CXCL family, IL-2, TNF-α, GM-CSF and IFN-γ. Similar findings were observed in conditioned media (CM) of purified PBMCs treated with ATG, where the increase in CCLs, CXCLs and other cytokines, including IFN-γ, was even more pronounced (Figure S3A).

6.2. ATG Induces IFN-γ Production and Release in $CD8^+$ Effector T Cell Subsets

As "cellular response to interferon-gamma" was the strongest regulated GO term and several of the identified upregulated cyto- and chemokines were IFN-γ-inducible, we next investigated the regulation and action of IFN-γ after ATG stimulation in more detail. First, we corroborated our findings from the proteome profiler with an IFN-γ ELISA and found that absolute plasma levels were significantly increased after treatment with ATG (575.5 ± 235.1 pg/mL; p-value = 0.0005) compared to the isotype and untreated control (both below the assays' detection limit, Figure 1F). Protein levels of IFN-γ in conditioned media of isolated PBMCs were also significantly higher in ATG-treated cells than in the controls (Untreated: 22.76 ± 22.76 pg/mL; Isotype: below assay detection limit; ATG: 575.0 ± 101.3 pg/mL, p-value = 0.0008) (Figure S3B). Only the subsets of effector $CD8^+$ T cells as well as the NK/T cells showed increased an expression of interferon-gamma mRNA (*IFNG*) following treatment with ATG (Figure 1G). Next, we aimed to further corroborate the IFN-γ modulating effects of ATG on $CD8^+$ T cells. Conditioned media of ATG-treated $CD8^+$ T cells revealed significantly higher levels for IFN-γ (3059 ± 173 pg/mL; p-value < 0.0001) as compared to the isotype (122 ± 47 pg/mL) and untreated controls (98 ± 28 pg/mL) after purification and stimulation (Figure 1H). Moreover, measurement of IFN-γ in $CD8^+$ T cell lysate immediately after isolation (218 ± 80 pg/mL) as well as 24 h after stimulation (untreated 24 h: 326 ± 35 pg/mL; isotype 24 h: 367 ± 56 pg/mL; ATG 24 h: 301 ± 45 pg/mL) showed no upregulation of IFN-γ levels by ATG, suggesting that newly produced IFN-γ is immediately released by the cells (Figure 1H). Together, our data indicate that ATG induces the production of IFN-γ in cytotoxic $CD8^+$ T cells, which in turn affects monocyte function.

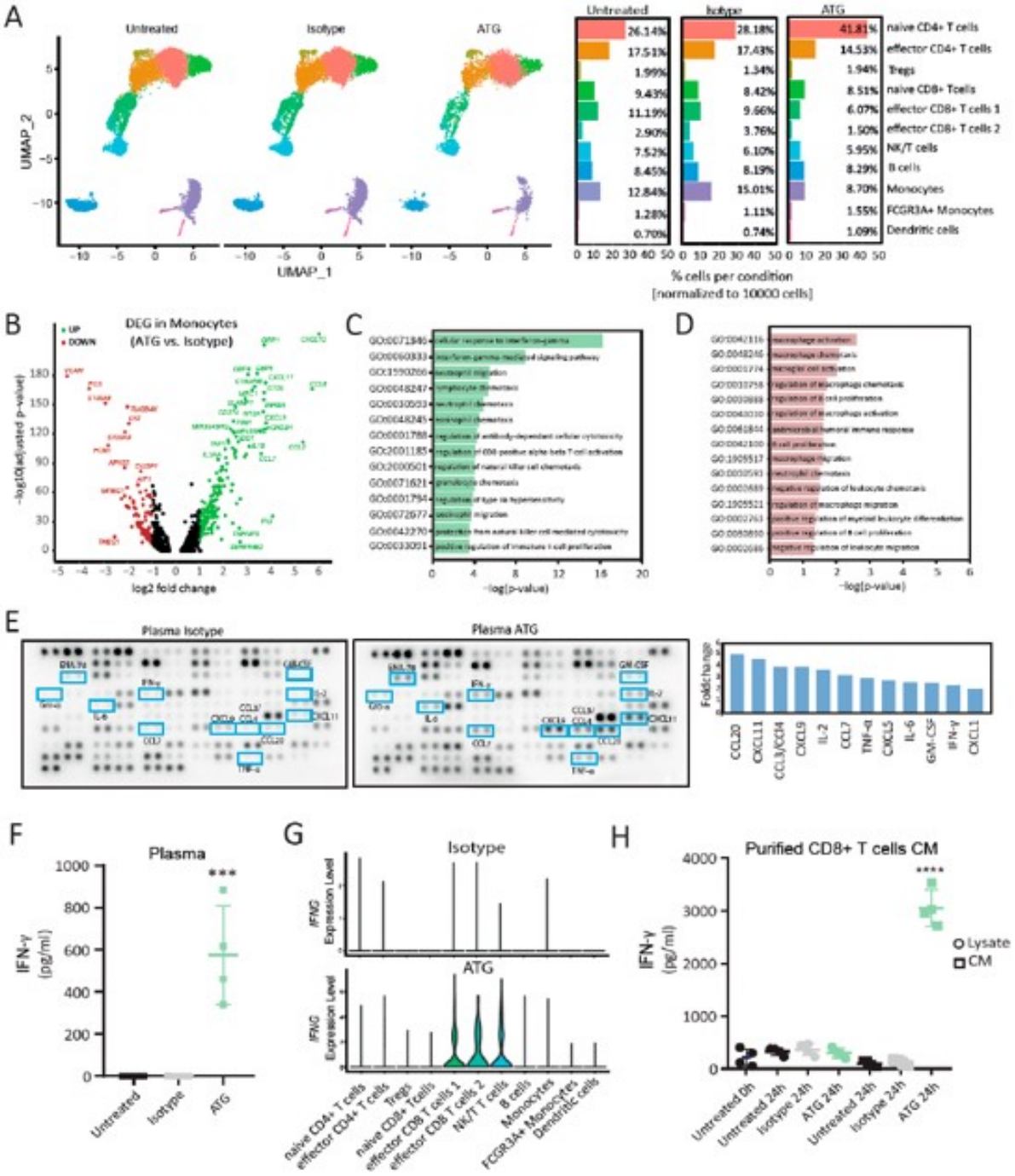

Figure 1. Single-cell RNA sequencing of ATG-treated white blood cells reveals transcriptional alterations in monocytes in response to IFN-γ. **(A)** Uniform manifold approximation and projection (UMAP) plot of untreated (Untreated), isotype-treated (Isotype) and ATG-treated (ATG) immune cells from whole blood reveals 11 distinct cell types present in all conditions. Relative distribution of each cell type across all investigated conditions is shown in the bar plots. N = 2 donors per condition. **(B)** Volcano plot showing differentially up- (green, $\log_2$FC > 1 and adj. *p*-value < 0.05) and downregulated (red, $\log_2$FC < −1 and adj. *p*-value < 0.05) genes (DEG) in monocytes (ATG vs. Isotype) with annotations. Adj. *p*-values were obtained after Benjamini–Hochberg correction.

Bar plot showing significantly overrepresented immune system processes associated with (**C**) up- and (**D**) downregulated genes in monocytes (ATG vs. Isotype). (**E**) Immunodetection array membrane of plasma of whole blood treated with ATG and the isotype control. Pooled supernatants of 3 donors per condition were analyzed. Proteins with a > twofold increase are shown in blue brackets and highlighted in the bar graph. (**F**) Quantification of IFN-γ by ELISA in plasma from untreated (black), isotype-treated (grey) and ATG-treated (green) whole blood. N = 4 donors. Asterisks denote *p*-values = 0.0005. Ordinary one-way ANOVA was performed followed by Dunnett's multiple comparisons. (**G**) Expression of *IFNG* across all clusters in the Isotype and ATG. Grade of gene expression is indicated by violin plot height while width represents proportion of positive cells. (**H**) Quantification of IFN-γ by ELISA in lysates (circle) and conditioned medium (square) of untreated (black), isotype-treated (grey) and ATG-treated (green) purified $CD8^+$ T cells. N = 4 donors. Asterisks denote *p*-values < 0.0001. Ordinary one-way ANOVA was performed followed by Dunnett's multiple comparisons.

6.3. Surface Expression of PDL-1 Is Increased on Monocytes of ATG-Treated Whole Blood

PDL-1, encoded by CD274, is known for its involvement in the regulation of T cell activity via the engagement of PD-1 [33]. In addition to several chemokines and cytokines, *CD274* was also significantly upregulated by ATG in monocytes, *FCGR3A*$^+$ monocytes and dendritic cells from whole blood (Figure 2A). Treatment with ATG resulted in a more than 2.5-$\log_2$-fold upregulation of CD274 expression in monocytes (Figure 2A). Almost 60% of all monocytes showed high levels of CD274 after ATG stimulation (0.32 ± 0.32% in untreated, 1.96 ± 1.613% in isotype-treated and 58.74 ± 10.01% in ATG-treated monocytes) (Figure 2B). These changes were further affirmed by the assessment of surface protein expression of PDL-1 in monocytes treated with 100 µg/mL ATG (Figure 3C). Twenty-four hours after stimulation of whole blood with ATG, 89.68 ± 5.2% (*p*-value < 0.0001) of monocytes were positive for PDL-1, while untreated (4.6 ± 0.5%) and isotype (6.6 ± 0.52%)-treated samples showed little, if any, PDL-1 expression (Figure 2C). Similarly, 88.9 ± 2.35% (*p*-value: < 0.0001) of monocytes in ATG-treated purified PBMCs were positive for PDL-1, as opposed to 2.105 ± 0.46% in untreated and 4.932 ± 0.76% in isotype control-treated PSMCs, respectively (Figure 2C). This effect was not observed at lower dosages of ATG (Figure S4). Together, these data suggest that ATG potently upregulates the expression of *CD274* on monocytes, which translates to an increase in the surface expression of PDL-1 on monocytes from whole blood and purified PBMCs.

6.4. IFN-γ Modulates Surface Expression of PDL-1 on Purified Monocytes

To identify the mechanism by which ATG increases PDL-1 expression in monocytes, we first investigated whether ATG-induced PDL-1 expression is a result of a direct or indirect stimulation of monocytes. For that reason, we purified monocytes from whole blood and stimulated them with ATG. The purity of isolated monocytes was assessed by flow cytometry and was above 95% (Figure S5A). Interestingly, direct stimulation of purified $CD14^+$ monocytes with ATG did not lead to an upregulation of PDL-1 expression (Figure 3A,B). Since our transcriptional data identified the strong regulation of several cytokines, we next investigated whether the modulation of PDL-1 was dependent on soluble factors induced by ATG. We therefore stimulated PBMCs with ATG for 24 h and added the resulting supernatant on purified monocytes. Indeed, monocytes incubated with conditioned media of ATG-treated PBMCs were strongly positive for PDL-1, while monocytes treated with conditioned media of untreated PBMCs showed almost no PDL-1 (Figure 3C,D; untreated PBMC SN: 4.44 ± 0.85%; isotype PBMC CM: 4.12 ± 0.78%; ATG PBMC CM: 89.9 ± 2.07%; *p*-value < 0.0001; for gating strategy, see Figure S5B). Furthermore, we evaluated the long-term prevalence of PDL-1^+ monocytes induced by the conditioned medium of ATG-treated PBMCs. Significantly higher mean percentages of PDL-1^+ monocytes were detected following treatment with ATG CM compared to the controls for up to 120 h (Figure S6). To investigate the signaling pathway(s) responsible for the observed ef-

fect, we next performed a gene set enrichment analysis of the genes regulated in monocytes by ATG. This analysis showed a significant enrichment of genes regulated by interferon-γ (Figures 3E and S7) and corroborated the top two immune system processes detected in our overrepresentation analysis (Figure 1C). In addition, we observed a significant regulation of the IFN-γ signaling pathway network between the effector *CD8*$^+$ T cell subsets and myeloid cell types, as shown by the chord diagram (Figure 3F), suggesting ligand–receptor communication based on the interactions of *IFNG* with its receptors *IFNGR1* and *IFNGR2*. To validate our bioinformatics data, we investigated the expression and function of the IFN-γ receptors on monocytes. Flow cytometry analysis confirmed the expression of IFN-γR1 and IFN-γR2 on purified monocytes (Figure S5C). The addition of IFN-γR1- but not IFN-γR2-blocking antibodies almost completely abolished PDL-1$^+$ upregulation in monocytes after treatment with PBMC-conditioned media (95.4 ± 1.58% vs. 16.07 ± 1.86%; *p*-value < 0.0001) (Figures 3H and S8). As the IFNγ signaling pathway network (Figure 3F) identified a possible interaction of T cells and NK cells with monocytes, we next isolated CD2$^+$ from PBMCs and stimulated them, as well as the CD2-depleted PBMC fraction with ATG. The CM of ATG-treated CD2$^+$ cells significantly increased the percentage of PDL-1$^+$ monocytes when compared to CM- or CD2-depleted PBMCs treated with ATG (89.4 ± 6.4% vs. 0.26 ± 0.2%, *p*-value = 0.002, Figure S9). These findings suggest that the engagement of IFNγR1 by IFN-γ is required to induce PDL-1 on purified monocytes.

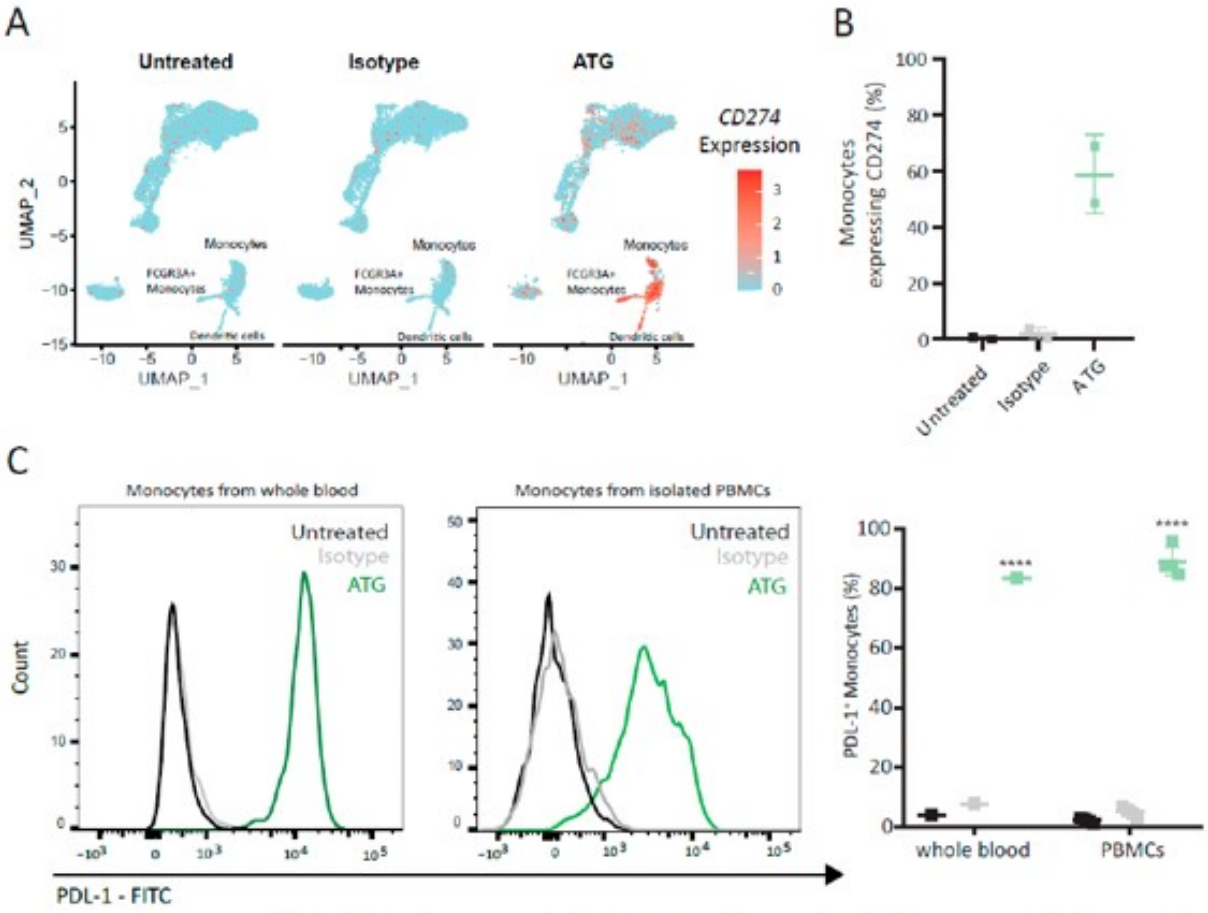

Figure 2. ATG upregulates *CD274* in monocytes from whole blood ex vivo. (**A**) Feature plot for *CD274* of untreated, isotype- and ATG-treated white blood cells. Monocytes, *FCGR3A*+ monocytes and dendritic cells are annotated and the red colour gradient indicates the expression level. (**B**) Bar

plot showing the mean percentage of monocytes expressing CD274 and standard error of the mean (SEM) in Untreated, Isotype and ATG. N = 2 donors. (C) Flow cytometry analysis for surface expression of PDL-1 on monocytes from whole blood and purified PBMCs after treatment with ATG. Histogram displaying percentage of PDL-1$^+$ monocytes in Untreated (black), Isotype (grey) and ATG (green) from whole blood and purified PBMCs. **** indicates p-value < 0.0001.

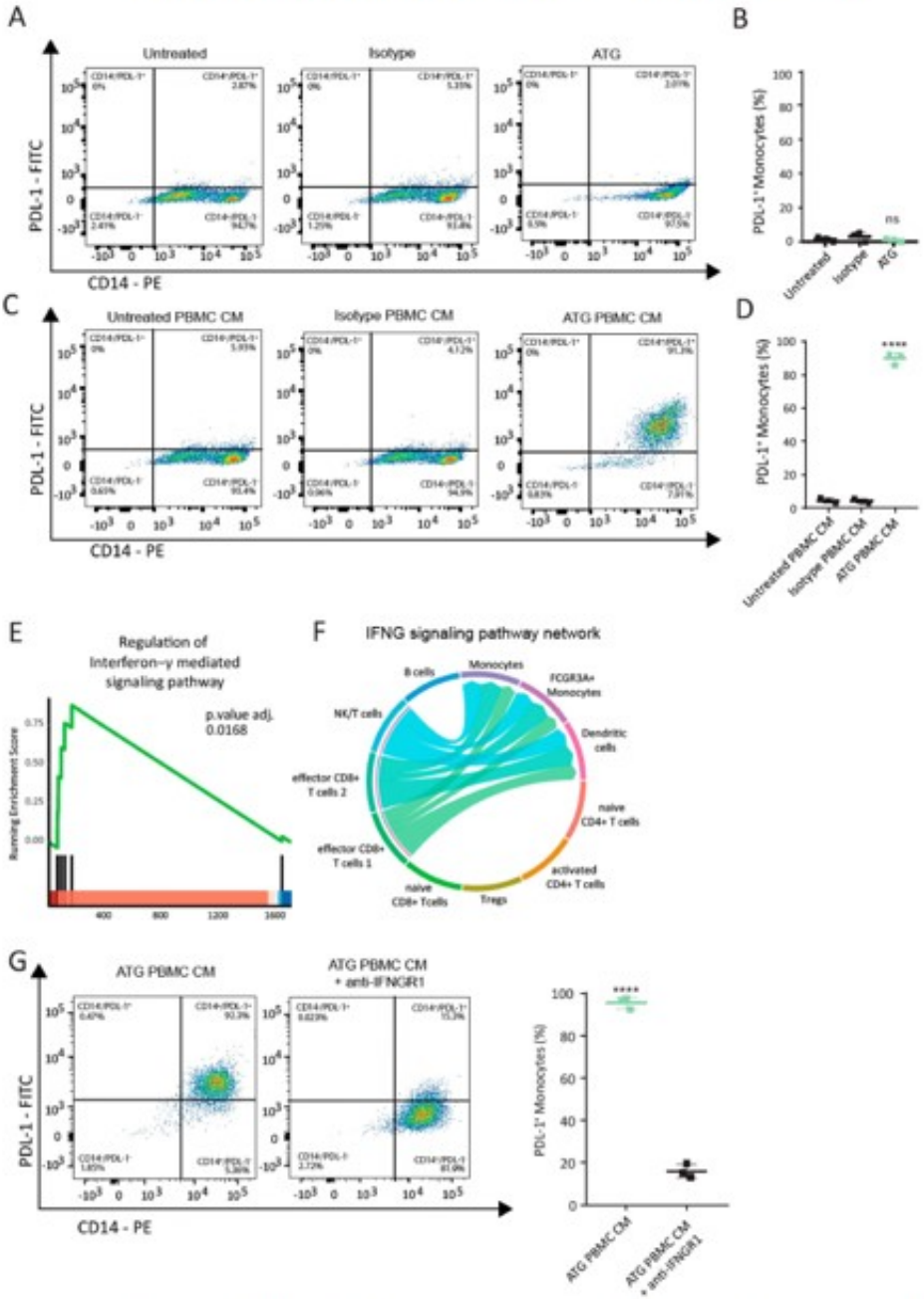

Figure 3. IFN-γ modulates upregulation of PDL-1 on purified monocytes. (**A**) Representative FACS plots of purified monocytes assessed for the surface expression of PDL-1 24 h after treatment with the isotype antibody or ATG. PDL-1$^+$ monocytes are displayed in the upper right quadrant. (**B**) Graph displaying the percentage of PDL-1$^+$ monocytes after the direct addition of the ATG and

controls. (C) Dot plots of purified monocytes stained for PDL-1 after treatment with conditioned media of untreated PBMCs, isotype-treated PBMCs and ATG-treated PBMCs and (D) graph displaying the percentages of PDL-1$^+$ monocytes. (E) GSEA enrichment plot for the representative signaling pathway enriched in monocytes from ATG-treated whole blood. The green line depicts the running enrichment score, and the black vertical lines indicate the rank of the genes responsible for the enrichment and position in the list of DEG. (F) Chord diagram of *IFNG* signaling pathway network in whole blood cells treated with ATG. (G) Representative FACS plots of purified monocytes treated with conditioned media of ATG-treated PBMCs. Surface expression of PDL-1 was assessed with prior inhibition of IFN-γR1 and without. Asterisks denote p-values < 0.0001 and were calculated by Student's t-test.

6.5. Inhibition of STAT1 by Ruxolitinib Abolishes Induction of PDL-1 on Monocytes by Paracrine Factors

Since IFN-γR1 signaling is known to involve the activation of the JAK/STAT pathway [34], we next investigated whether the activation of STAT1 influences PDL-1 expression on monocytes. *STAT1* mRNA expression was upregulated in monocytes, *FCGR3A*$^+$ monocytes and dendritic cells (Figure S1E). Moreover, we detected a significant enrichment of the receptor signaling pathway via JAK/STAT (adjusted p-value: 0.0022) in monocytes of ATG-treated whole blood (Figure 4A). Further investigation of the JAK/STAT gene family members involved in the key enrichment of this pathway revealed the strong upregulation of *JAK2*, *STAT1*, *STAT2 and STAT3* in monocytes (Figure 4B), but also other cell types of ATG-treated whole blood (Figure S1O). In contrast to the controls, treatment with conditioned media of ATG-treated PBMCs increased phosphorylation of STAT1 in purified monocytes (Figure 4C). Pre-incubation of purified monocytes with the JAK inhibitor ruxolitinib strongly reduced this effect (Figure 4C). Importantly, the addition of ruxolitinib to monocytes treated with conditioned media of ATG-treated PBMCs strongly inhibited PDL-1$^+$ expression (Figure 4D). While 92.23 ± 3.06% of monocytes incubated with ATG PBMC CM were positive for PDL-1, only 4.97 ± 1.84% of monocytes were positive for PDL-1 when cells were pretreated with ruxolitinib (Figure 4E, p-value < 0.0001). Taken together, we show that the modulation of PDL-1 surface expression on monocytes by ATG is regulated by IFN-γ and dependent on its binding to IFN-γR1 and downstream activation of the JAK/STAT signaling pathway.

6.6. PDL1$^+$ Monocytes Inhibit CD8$^+$ T Cell Proliferation and Release of Granzyme B In Vitro

Activation of co-stimulatory and co-inhibitory signals influences effector functions of T lymphocytes [35]. The binding of PDL-1 to PD-1 confers an inhibitory signal [36]. Monocytes in the ATG condition displayed a positive enrichment for biological processes associated with the regulation of CD8$^+$ T cell activation (adjusted p-value: 0.0124), negative regulation of T cell proliferation (adjusted p-value: 0.0086) and tolerance induction (adjusted p-value: 0.0086) (Figure 5A). *CD274*, *IDO1*, *IRF1*, *HLA-A* and *HLA-E* were identified as core enrichment genes for these processes (Figure 5B). Therefore, we thought to assess whether PDL-1$^+$ monocytes are able to suppress effector functions of activated CD8$^+$ T cells in vitro. Co-cultures of PDL-1$^+$ monocytes with αCD3/αCD28-activated CD8$^+$ T cells significantly reduced their proliferative capacity (Figure 5C; for gating strategy, see Figure S4D). When incubated with untreated control monocytes, 55.03% ± 2.37% of CD8$^+$ T cells divided ≤ 3 times and 44.98% ± 2.37% divided ≥ 4 times after 5 days (Figure 5D,E). In contrast, 81.63% ± 2.98% of activated CD8$^+$ T cells divided ≤ 3 times and 18.38 ± 2.98% divided ≥ 4 times (Figure 5D,E; p-value = 0.0009) after co-culture with PDL-1$^+$ monocytes. This effect was significantly less pronounced when PDL1$^+$ monocytes were pretreated with durvalumab, a monoclonal antibody directed against PDL-1, prior to co-culture with activated T lymphocytes (59.78 ± 4.44% displayed ≤ 3 and 40.23 ± 4.44% ≥ 4 divisions (Figure 5D,E; p-value = 0.0034). In contrast to CD8$^+$ T cells, the addition of CM from ATG-treated PBMCs did not inhibit the proliferation of activated

CD8⁺ T cells when co-cultured with PDL1⁺ monocytes (percentage of cells with four or more divisions 74.85 ± 3.71%; *p*-value = 0.0002) (Figure S11). Next, we also measured the protein levels of the effector cytokine granzyme B in supernatants of activated CD8⁺ T cells co-cultured with monocytes. The addition of PDL-1⁺ monocytes resulted in significantly lower levels of granzyme B (7.01 ± 0.58 ng/mL) when compared to the untreated controls (11.41 ng/mL ± 0.79; *p*-value = 0.0046) and anti-PDL-1 antibody pretreated PDL-1⁺ monocytes (10.96 ± 0.26 ng/mL; *p*-value = 0.0077) (Figure 5F). Together, these data show that PDL-1⁺ monocytes inhibit the effector functions of activated CD8⁺ T cells, resulting in reduced T cell proliferation and granzyme B secretion.

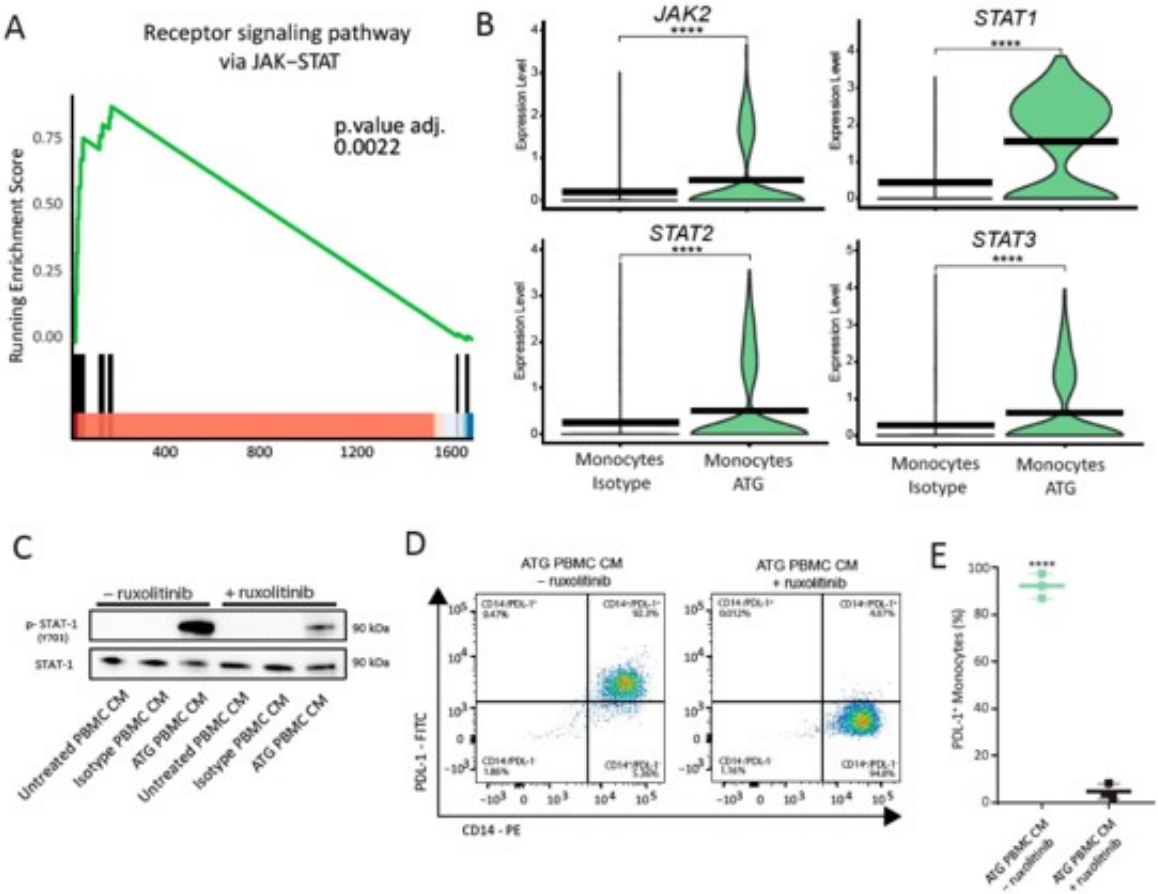

Figure 4. STAT-1 activation is required for the induction of PDL-1 on purified monocytes. (**A**) GSEA enrichment plot for the receptor signaling pathway via JAK-STAT in monocytes from ATG-treated whole blood. The green line depicts the running enrichment score, and the black vertical lines indicate genes responsible for the enrichment and position in the ranked list of DEG. (**B**) Violin plots depicting the differential expression of *JAK2*, *STAT1*, *STAT2* and *STAT3* in monocytes following stimulation with ATG. Expression levels are indicated by violin plot height, while width represents the proportion of positive cells. Crossbars mark mean expression. **** indicates *p*-value < 0.0001. (**C**) Representative Western blots of purified monocytes pretreated with the JAK/STAT inhibitor ruxolitinib for 1 h before stimulation with conditioned media of ATG-treated PBMCs and controls. The levels of total STAT1 and phosphorylated STAT1 were assessed after 2 h. (**D**) FACS plots showing the percentage of monocytes positive for PDL-1 24 h after the addition of conditioned medium of ATG-treated PBMCs with and without the prior addition of ruxolitinib. (**E**) Quantitative analysis for surface expression of PDL-1 on monocytes in the investigated conditions for N = 3 donors. Asterisks denote *p*-values < 0.0001. Student's *t*-test was used to determine significance.

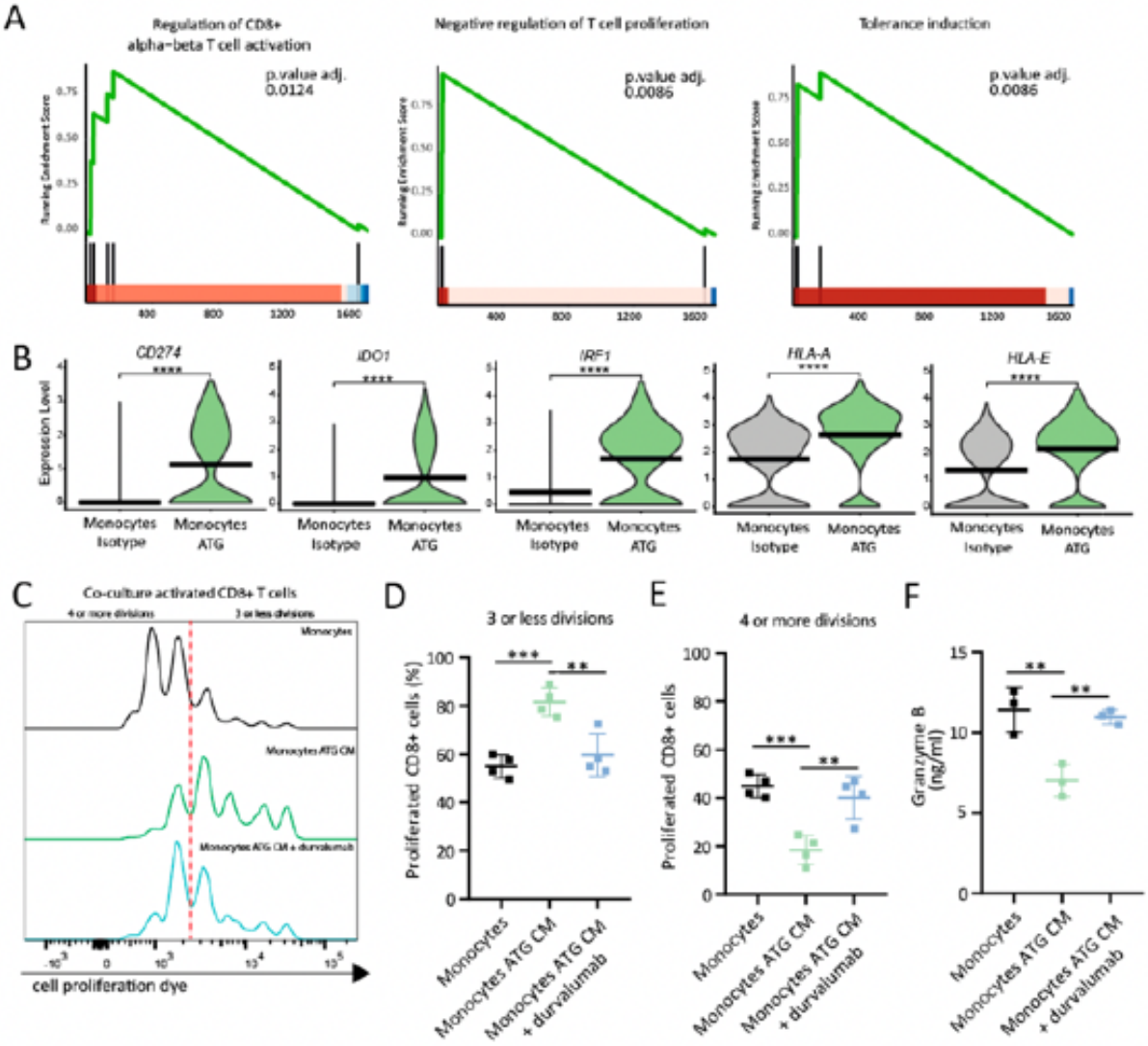

Figure 5. PDL-1$^+$ monocytes reduce proliferation and granzyme B release of activated CD8$^+$ T cells. **(A)** GSEA enrichment plot for the regulation of T cell functions and tolerance induction in monocytes following treatment with ATG. The green lines depict the running enrichment score, and the black vertical lines indicate the genes responsible for the enrichment and position in the ranked list of DEG. **(B)** Violin plots of core enrichment genes *CD274*, *IDO1*, *IRF1*, *HLA-A* and *HLA-E* in monocytes treated with the isotype control vs. monocytes treated with ATG. Expression levels are indicated by violin plot height, while width represents the proportion of positive cells. Crossbars mark mean expression. **** indicates p-value < 0.0001. **(C)** Representative histogram of the proliferative capacity of activated CD8$^+$ T cells co-cultured with monocytes (black), PDL-1$^+$ monocytes (green) and durvalumab-pretreated PDL1$^+$ monocytes (blue). Non-proliferating (furthest right) and proliferating cell populations are reflected by the intensity of cell proliferation dye staining. The dashed red line separates cells with 3 or less divisions from cells with 4 or more divisions. **(D)** Bar graph depicting the mean percentages of CD8$^+$ T cells with 3 or fewer proliferations and **(E)** 4 or more proliferations on day 5. N = 4 donors per group. *** p-value = 0.0009; ** p-value = 0.0034. One-way ANOVA with Dunnett's multiple comparison was used to determine statistically significant differences. **(F)** Concentration of granzyme B in a conditioned medium of CD8$^+$ T cells co-cultured with monocytes assessed by ELISA. ** p-value < 0.01.

7. Discussion

The immunosuppressive action of ATG is generally attributed to its T cell-depleting properties [7,9,13]. However, increasing evidence from several groups suggests additional modes of action, mediated either directly on distinct T lymphocyte subsets or indirectly via the release of paracrine factors [12,37–39]. Using scRNA-seq, we detected numerous alterations in gene expression across lymphoid and myeloid cell types of white blood cells from human whole blood treated with ATG. Notably, classical monocytes displayed significant changes that were tightly connected to responses to IFN-γ. As a result, the surface expression of PDL-1 was increased in these cells, enabling them to functionally impair the proliferative capacity and release of granzyme B of activated $CD8^+$ T cells, representing an additional mechanism by which ATG exerts its immunosuppressive actions.

Here, we provide a broad overview of the transcriptional changes modulated by the ex vivo treatment of human whole blood with ATG, capturing different subsets of lymphoid and myeloid cell types. Transcriptional changes in the lymphoid cells are explainable by the antigen specificity of ATG [40]. Influences on myeloid cell types such as natural killer cells and monocyte-derived dendritic cells have been previously reported by others [17,38]. Dalle et al. showed the increased production of IFN-γ in natural killer cells [38], while Roider et al. reported on the in vitro induction of tolerogenic dendritic cells by ATG [37]. We and others previously showed that PBMCs treated with ATG show an increased production of IFN-γ [41,42]. In line with these reports, our current analysis revealed an upregulation of *IFNG* in NK/T cells after stimulation with ATG. Beyond that, we observed the increased expression of *IFNG* in the subsets of effector *CD8*$^+$ T cells following treatment with ATG and identified these to be specifically modulated by ATG to increase the production and release of IFN-γ. While IFN-γ is generally considered as a pro-inflammatory cytokine, several reports suggest immunosuppressive roles for IFN-γ [43]. The priming of human mesenchymal stem cells with IFN-γ modulated their immunosuppressive capacity and resulted in clinical improvement and prolonged survival in a murine model of graft vs. host disease [44]. Furthermore, IFN-γ has been shown to confer immunosuppressive actions by modulating the expression of the inhibitory immune receptor ligand PDL-1 on various cell types [45–47]. In addition to IFN-γ, numerous other cyto- and chemokines were induced by ATG, including in particular, members of the CCL and CXCL family as well as immunomodulatory cytokines. CXCL9, CXCL10 and CXCL11 have especially been considered as IFN-γ response cytokines and have been reported to modulate the expression of PDL-1 in gastric cancer cells [48]. Their presence might explain why after the inhibition of IFN-γR1, the induction of PDL-1 on monocytes was not completely abolished. Furthermore, these factors bind to the chemokine receptor CXCR3 and transduce their signal via the activation of the JAK/STAT pathways [49], supporting the superior inhibition of PDL-1 induction by the JAK/STAT inhibitor used in our experiments. The overrepresentation of paracrine factors regulated by ATG and the resulting activation of intracellular signaling pathways highlight their relevance in the effects mediated by ATG. Our data further substantiate the importance of paracrine factors released by white blood cells in response to ATG and describe an additional immunosuppressive mechanism beyond the already known depletion of T cells.

The observed increase in the surface expression of PDL-1 on monocytes was mediated by the paracrine signaling of IFN-γ and led to an inhibitory effect on activated $CD8^+$ T cells. This mechanism has previously been described as an evasion mechanism of different tumor entities [50,51]. Binding of IFN-γ to IFN-γR1 modulated the expression of PDL-1 on tumor cells and suppressed anti-tumor host immunity, leading to disease progression, increased dissemination and a worse overall outcome [52]. While these regulations are clearly detrimental in oncologic settings, following transplantation, modulation of the PD1:PDL-1 axis can significantly improve graft survival and peripheral tolerance [53]. Pre-clinical work by Borges et al. revealed a significant increase in the graft tolerance of PD-1 overexpressing T cells in a fully MHC-mismatched murine cardiac transplantation model [54]. Interestingly, transplantation of PDL-1 knockout donor hearts into PD-1-overexpressing mice resulted

in immediate graft rejection, strongly underlining the importance of the receptor–ligand pair for peripheral tolerance [54]. Moreover, the expression of PDL-1 can be found in other cell types besides myeloid cells. Recently, Bracamonte-Baran et al. reported the increased presence of PDL-1 in graft endothelial cells and their inverse correlation with the infiltration of $CD8^+$ T lymphocytes in the myocardial biopsies of heart transplant recipients [55]. Furthermore, they showed that in fully MHC-mismatched mice, a conditional knockout of PDL-1 in graft endothelial cells significantly decreased graft survival when compared to the PDL-1-expressing controls [55]. Our observations are restricted to immune cells from whole blood. Still, ATG and paracrine factors induced by ATG might also affect other cell types found in the vasculature or even in the transplanted organ and similarly modulate PDL-1 or alternative pathways to contribute to graft tolerance by reducing T cell-mediated tissue damage.

The emergence of immune therapeutic drugs for the targeted inhibition of co-stimulatory signals with inhibitory effects on the activation and proliferation of T cells, i.e., PDL-1 and PD-1, amongst others, has advanced the treatment of patients affected by oncologic malignancies [56]. However, severe adverse effects related to excessive activation of the immune system by therapy with immune checkpoint inhibition have been reported in these patients [57]. These immune-related adverse events most commonly involved hepatitis, colitis, myocarditis and dysregulations of endocrine systems including the pancreas and adrenal glands [58]. Interestingly, several reports showed that ATG is able to counteract adverse events caused by immune checkpoint inhibition [59–61]. While this effect is in part explainable by its T cell-depleting properties, based on our data, it is tempting to speculate that the induction of PDL-1 on monocytes, or other cell types, potentially contributes to the inhibition of autoreactive T cells, resulting in an amelioration of the reported adverse effects.

Moreover, transplanted patients are naturally at a higher risk of developing neoplasia due to ongoing maintenance immunosuppressive therapy [62,63]. While there are no large randomized clinical trials available, several case reports described a high risk of graft failure and rejection in solid organ transplant recipients treated with immune checkpoint inhibitors underlining the importance of the PD1:PDL-1 axis for the maintenance of peripheral graft tolerance [64,65]. Whether ATG might prove beneficial in these specific settings, where fine margins between the maintenance of graft survival are confronted with the requirement of anti-tumor immunity, has to be addressed in future studies. Our data show that ATG is able to increase the levels of PDL-1 on monocytes and other myeloid blood cells, and the interaction of these cells with activated T cells results in the reduction in their effector functions, including the proliferation and release of granzyme B.

Our findings are limited to pre-clinical ex vivo and in vitro data. Studies addressing the role of ATG-induced PDL-1 in myeloid cells and its relevance in patient settings will be needed to clarify to what degree this effect contributes to ATG-mediated graft tolerance. In addition, such studies will be better suited to investigate the influences of repeated administrations of ATG, as performed in standardized induction protocols, and enable a prolonged observation of the described effects. Nevertheless, our data indicate a prolonged positivity of PDL-1 on monocytes treated with a single administration of a conditioned medium of ATG-treated PBMCs. In addition, immunosuppressive induction therapy in patients with high immunological risk of graft rejection usually includes additional drugs such as calcineurin inhibitors, mycophenolate-mofetil and glucocorticoids [66]. Of note, pretreatment of PBMCs with an immunosuppressive dose of hydrocortisone prior to the addition of ATG did not decrease PDL-1 on monocytes (Figure S12). This suggests that the combined use of these agents does not interfere with PDL-1 induction. Further studies are needed to reliably dissect potential interactions between these and other compounds regularly used for immunosuppression in combination with ATG. In conclusion, our study provides a comprehensive overview of the transcriptional changes in immune cells from human whole blood treated with ATG on a single cellular level. We identified an increase in the surface expression of PDL-1 on monocytes as a result of paracrine signaling

via IFN-γ/STAT/JAK. Additionally, we showed that PDL-1$^+$ monocytes potently inhibit the effector functions of activated CD8$^+$ T cells in vitro. Altogether, our data suggest an additional mechanism by which ATG indirectly modulates immunosuppressive actions on cell-mediated rejection in white blood cells.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/cells12030382/s1, Figure S1. Comparison of differential responses of cytotoxic *CD8*$^+$ T cells and myeloid cells; Figure S2. Differential gene expressions of immune cells from human whole blood treated with ATG; Figure S3. Cytokine profiling of PBMCs treated with ATG. Figure S4. Percentages of PDL-1 on monocytes from purified PBMCs in response to different doses of ATG. Figure S5. Gating strategy for flow cytometry experiments; Figure S6. Increased surface expression of PDL-1 on monocytes monitored over time; Figure S7. Leading enrichment genes for regulation of IFN-γ-mediated signaling pathway in monocytes treated with ATG; Figure S8. Blockage of IFN-γR2 does not prevent induction of PDL-1 on monocytes stimulated with conditioned medium of ATG-treated PBMCs; Figure S9. Conditioned media of CD2$^+$ cells treated with ATG increases PDL-1 on purified monocytes; Figure S10. Differential regulation of JAK/STAT pathway members across lymphoid and myeloid immune cells after treatment with ATG; Figure S11. Proliferation of active CD8$^+$ T cells is not inhibited when compared to co-cultures with PDL-1$^+$ monocytes; Figure S12. Pretreatment of PBMCs with hydrocortisone does not prevent induction of PDL-1 by ATG; Table S1. Overview on used antibodies.

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study. Written informed consent has been obtained from the patient(s) to publish this paper.

Data Availability Statement: Except for the scRNA-seq experiments, all data are available in the main text or in the supplementary materials. The scRNA-seq data are accessible upon request.

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Conflicts of Interest: The authors declare that they have no competing interests.

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CHAPTER THREE: DISCUSSION

The secretome of irradiated peripheral blood mononuclear cells (PBMCsec) and anti-thymocyte globulin (ATG) distinctively modulate immunological functions through secondary paracrine effects. The findings presented in this thesis reveal novel immunomodulatory actions for PBMCsec and ATG that delineate not only from direct actions, but more precisely by induction of secondary mediators. This paradigm of extension of the mechanistic properties of a medicinal product by induction and release of additional endogenous factors from a broad scope of target cells stands in contrast to conventional single-target therapies (Petrelli & Giordano, 2008).

PBMCsec transcriptionally reprograms immune cells towards a regenerative phenotype with the subsequently amplified release of paracrine factors. Thereby PBMCsec promotes the release of a secondary secretome with congruent as well as distinct functional properties to original PBMCsec.

For ATG, the presented findings expand the existing knowledge of its immunosuppressive mechanisms of action. We provide evidence for alternative immunosuppressive actions beyond T cell depletion by induction of immune checkpoints in myeloid cell populations. Precisely, ATG stimulates immune cells to secrete cytokines that upregulate the co-inhibitory ligand PD-L1 on monocytes, which in turn engages with PD-1 on effector cells to potently dampen their responses.

Together, these insights highlight novel modes of action for both therapies, positioning them as immunomodulators that act in part through secondary paracrine mechanisms. The subsequent sections will focus on mechanistic and clinical implications of these findings. Additionally, findings will be positioned within the extensive landscape of immunomodulatory research and put into perspective for potential future avenues.

3.1 PBMCsec – primary and secondary wave of paracrine actions

PBMCsec has demonstrated regenerative effects in numerous preclinical studies on tissue repair (Beer *et al.*, 2016) and reinforced transition to clinical trials (Gugerell *et al.*, 2021; Simader *et al.*, 2017). Here we add to previously conducted studies on immunomodulatory properties of PBMCsec (Klas *et al.*, 2022; Laggner *et al.*, 2022; Laggner *et al.*, 2020a) by in-depth characterization of transcriptional alterations resulting from stimulation of immune cells from human whole blood *ex vivo*.

Single-cell RNA sequencing of human whole blood treated with PBMCsec revealed widespread gene modulation across multiple leukocyte subsets with classical monocytes displaying the most pronounced changes in comparison to minor alterations detected in lymphocyte populations. Monocytes responded to PBMCsec with an upregulation of 322

genes, including a plethora of regenerative and immunomodulatory mediators. The predominant responses observed in monocytes are consistent with their physiological role as rapid responders of innate immunity and fall in line with previous reports of immunomodulatory responses to extracellular vesicles and paracrine factors from adipose tissue derived mesenchymal stem cells (Beez *et al*, 2019; Guillén *et al*, 2018). Notably, our data revealed strongly induced transcripts for pro-angiogenic factors such as VEGFA, CXCL1, and CXCL5, consistent with the previously reported pro-angiogenic and tissue-healing properties of PBMCsec (Ankersmit *et al.*, 2009; Lichtenauer *et al.*, 2011b; Wagner *et al*, 2018).

Moreover, PBMCsec induced transcriptional changes significantly enhanced gene signatures associated with inhibition of endopeptidase activity in monocytes, exemplified by upregulation of SERPINB2. SERPINB2 – gene transcript translates to plasminogen activator inhibitor-2 (PAI-II/SerpinB2) - is known to be one of the most upregulated proteins in activated monocytes during inflammation, suggesting a highly conserved response (Major *et al*, 2013; Schroder *et al*, 2010). *In vivo* studies support a protective role in experimental ischemia-reperfusion models of kidney injury in mice (Sen *et al*, 2020) as well as macrophage function during enteric infection (Shea-Donohue *et al*, 2014). In these settings PAI-II aided in the resolution of inflammation by enhancing monocyte/macrophage retention and augmented phagocytosis at injury sites while limiting excessive tissue damage (Sen *et al.*, 2020). Excessive activity of inflammatory protease activity is an established contributor to endothelial barrier dysfunction in disease (Jerke *et al*, 2015; Minami *et al*, 2004). Therapeutic approaches directed towards inflammatory proteases targeting cathepsin A, neutrophil elastase, trypsin, and urokinase showed convincing success in experimental models of myocardial infarction (Mauro *et al*, 2017; Ogura *et al*, 2021; Petrera *et al*, 2016) and skin fibrosis (Vorstandlechner *et al*, 2021).

In our experiments SERPINB2 was the highest upregulated transcript in PBMCsec-treated monocytes marked by the greatest positive $\log_2$ fold-change of all differentially expressed genes in monocytes. Furthermore, its robust induction translated to high protein levels and had functional implications in suppression of serine-protease activity of thrombin and urokinase in independent *in vitro* assays. Of note, plasma from PBMCsec-treated blood potently inhibited the serine protease urokinase in an *in vitro* assay assessing endothelial barrier function. The fact that PBMCsec itself contains negligible urokinase inhibitory activity indicates that this anti-proteolytic effect is relayed by de novo synthesis of SerpinB2. This finding puts major emphasis on the fact that PBMCsec not only supplies a mixture of tissue-regenerative factors (Beer *et al*, 2015) but also affects the host's immune cells to generate additional molecules that extend the mechanistic profiles of PBMCsec.

By induction of SerpinB2, PBMCsec effectively triggers a sequential wave of anti-protease defense to add to the already broad spectrum of mechanistic actions of PBMCsec (Beer *et al.*, 2016). Beyond SerpinB2, stimulation with PBMCsec led to the release of a broad array of secondary cytokines and growth factors. Genes such as EREG (epiregulin), IL1B (interleukin-1β), CXCL1, and CXCL5 were consistently upregulated in monocytes upon treatment with PBMCsec. This profile is indicative of a complex pattern of immune activation. Epiregulin is a member of the EGF family implicated in tissue repair and regeneration (Draper *et al*, 2003), IL-1β is a potent pro-inflammatory cytokine that can also drive healing responses in controlled contexts (di Giovine *et al*, 1991), and CXCL1/CXCL5 are chemokines that recruit neutrophils to injured tissues and support angiogenesis (Russo *et al*, 2014). At first, the induction of a pro-inflammatory cytokine like IL-1β alongside anti-proteases and growth factors might seem paradoxical. However, effective tissue regeneration requires a coordinated inflammatory stimulus to initiate repair, followed by anti-inflammatory and matrix-protective signals to resolve healing without fibrosis (Eming *et al*, 2014). These aspects apply to PBMCsec. On the one hand, it transiently activates innate immune cells while simultaneously promoting protective mechanisms – including growth factors and chemokines to support the initial response as well as additional induction of SerpinB2 – to synergistically abrogate tissue damage. This multi-facetted immunomodulatory action highlight monocytes as host amplifiers of PBMCsec's effects via modulation of a secondary paracrine factor pool.

As such, monocyte-derived Vegf-A and Cxcl1/Cxcl5 act on endothelial cells to spur angiogenesis and tube formation. This coordination between induction of factors that result in cell recruitment and growth factor release in combination with the anti-proteolytic effects restraining overshooting collateral damages represents a structured sequence of paracrine actions supporting tissue regeneration (Lee *et al*, 2005; Makarova *et al*, 2011). However, the ability to harness monocytes as paracrine mediators is not restricted to PBMCsec. Various protective effects mediated by conditioned media of different cell sources have been identified as potent modulators of monocyte functions (Bormann *et al*, 2023). As such soluble factors from adipose-tissue derived MSC confer paracrine anti-inflammatory actions by reduction of pro-inflammatory mediators and prompting M2-polarization (Guillén *et al.*, 2018; Li *et al*, 2021). Use of conditioned media from transiently stressed PBMCs by exposure to hypoxia or oxygen-glucose-deprivation (OGD) has similarly resulted in upregulation of pro-survival and cytoprotective pathways in models of ischemic injury (Hatakeyama *et al*, 2019).

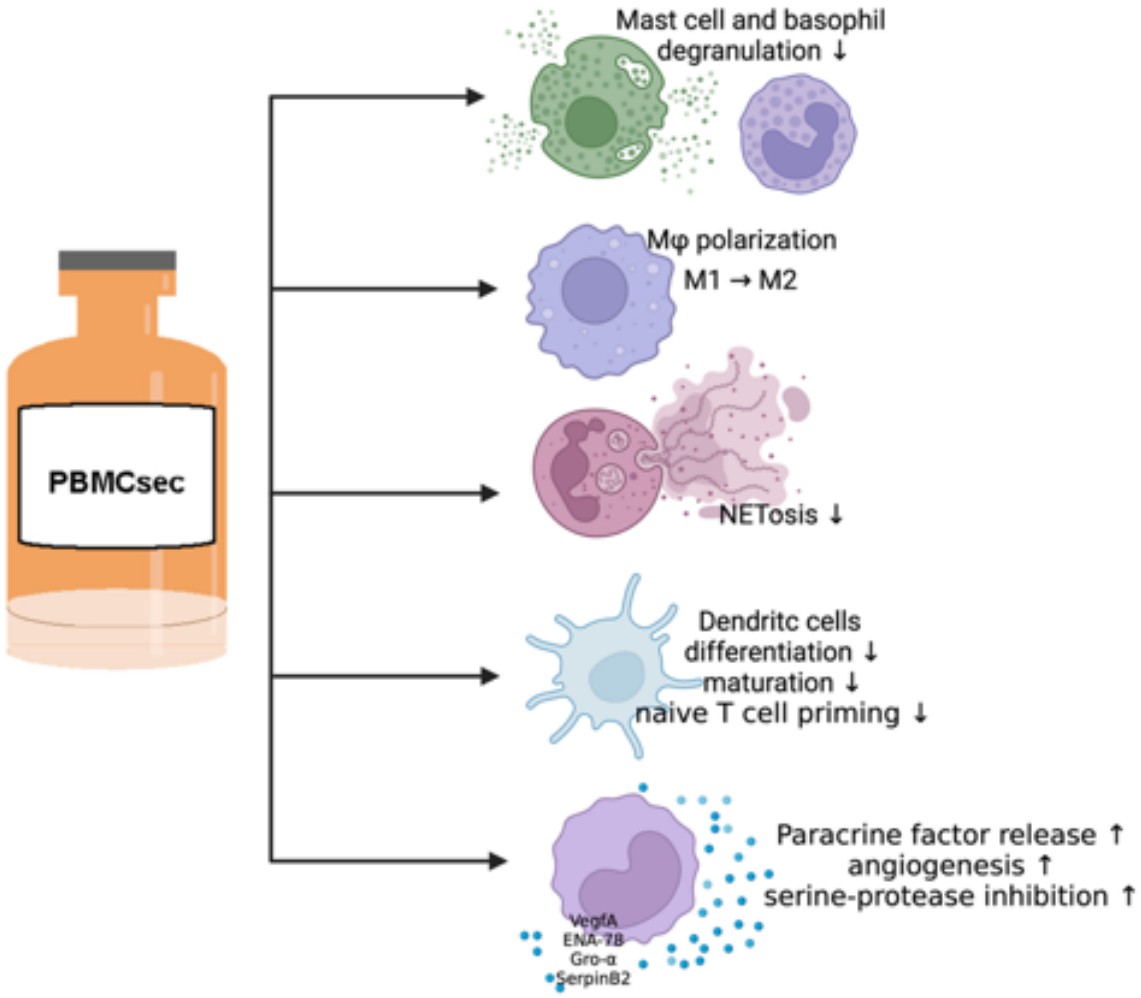

Figure 5. Effects of PBMCsec on immune cell functions. Created in https://BioRender.com

The induced secondary secretome is a result of the complex and diverse mix of functionally active components within PBMCsec which include different protein and lipid classes as well as extracellular vesicles (Beer *et al.*, 2015). This underscores that PBMCsec is not a single-factor therapy but a multifactorial agent with the ability to engage multiple pathways simultaneously.

Prior work has shown that all these components coordinate their actions to maximize PBMCsec's biological effects. As such, Laggner et al. demonstrated that lipids within the PBMCsec interfere with dendritic cell maturation and inhibit basophil/mast cell degranulation resulting in diminished skin inflammation and allergic responses (Laggner *et al.*, 2022; Laggner *et al.*, 2020a). Our current findings extend this concept by showing that PBMCsec's impact on monocytes is a key facet of its mode of action in human immune cells following *ex vivo* stimulation. Moreover, PBMCsec changes the functional phenotype of monocytes by favoring the secretion of additional regenerative and protective factors. This phenomenon provides a compelling explanation for the previously reported potency and durability of PBMCsec's effects captured in previous *in vivo* studies. For instance, a single intravenous

dose of PBMCsec was reported to almost completely abrogate myocardial injury in a porcine infarction model, even though direct pharmacokinetic tracking showed PBMCsec's components persisted only for a few hours in the bloodstream (Lichtenauer *et al.*, 2011b). Our data suggest that this discrepancy can be resolved by the relayed effect of the host immune system most prominently involving monocytes. Thereby, PBMCsec's short-lived presence might suffice to trigger prolonged production of secondary mediators resulting in a cascade of paracrine signaling extending beyond the initial stimulus.

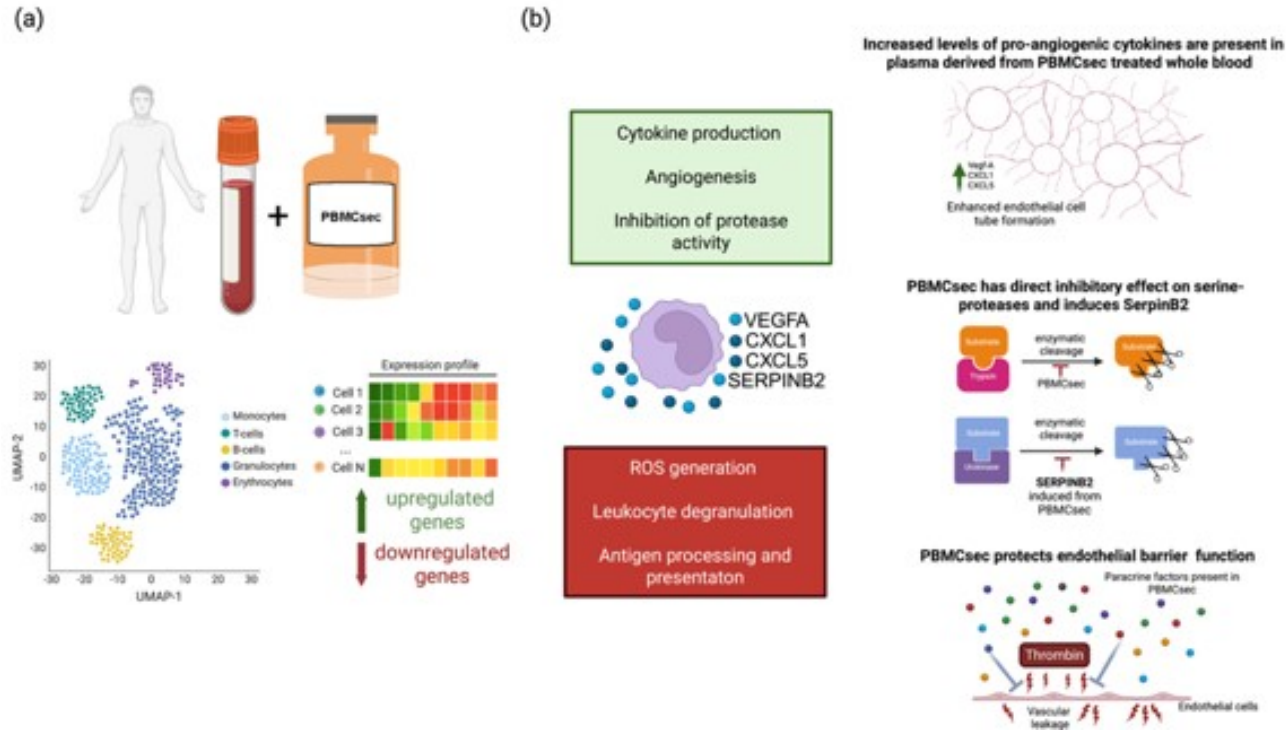

Figure 6.: Graphical Summary: PBMCsec alters monocytes functions to promote angiogenesis, protease inhibition and protection of endothelial barrier function.
(a) Ex vivo treatment of human whole blood with PBMC-derived secretome (PBMCsec) followed by single-cell transcriptomics (UMAP shown) identifies major cell populations and reveals cell-type- specific differential gene expression in response to PBMCsec.
(b) Pathway analysis shows up-regulation (green) of cytokine production, angiogenesis, and protease-inhibitory activity and down-regulation (red) of ROS generation, leukocyte degranulation, and antigen processing/presentation. Monocytes treated with PBMCsec release paracrine factors that are present in PBMCsec as well as de novo factors to expand the profile of tissue-protective factors. Functionally, plasma from PBMCsec-treated blood displays elevated pro-angiogenic cytokines that support endothelial cell tube formation. PBMCsec directly inhibits serine proteases (e.g., trypsin) and induces the urokinase inhibitor SERPINB2 and PBMCsec preserves endothelial barrier integrity against thrombin-induced leakage. Created in https://BioRender.com

3.2 ATG: Beyond T-Cell Depletion – Induction of PD-L1 for immune tolerance

ATG has long been used in transplantation medicine for its potent T-cell depleting capabilities (Mohty, 2007), but the herein presented findings elucidate a novel immunoregulatory mechanism complementary to its cytolytic action. Accumulating evidence has uncovered effects beyond lymphocyte depletion for ATG. As such, low-dose ATG has been demonstrated to expand CD4+CD25+FoxP3+ T_{regs} *in vitro* and *in vivo,* suggesting that ATG may shift the prevalent T-cell pool towards a more tolerogenic composition (Lopez *et al*, 2006). Additionally, ATG affects APCs by binding to antigens on dendritic cells to modulate their function (Leitner *et al*, 2011). Prior studies reported that ATG exposure inhibits monocyte-to-dendritic cell differentiation and drives DCs to a tolerogenic phenotype characterized by IL-10 production and expression of indoleamine 2,3-dioxygenase (IDO) (Roider *et al.*, 2016). Presence of shared antigens across different immune cell populations and diverse antigen specificities of polyclonal ATG explain the effects on different immune cell populations (Popow *et al.*, 2013).
Our findings complement and expand on these reports by uncovering large scale transcriptional modulations across various immune cell populations and identifying monocytes as crucial target population of ATG and its secondary effects. We demonstrate that exposure to ATG leads to monocyte-mediated immunomodulation via the PD-1/PD-L1 axis. Thereby, myeloid cells acquire a phenotype that allows them to actively suppress T-cell responses. Transcriptomic and confirmatory plasma protein measurement from ATG-treated whole blood revealed elevated levels for a broad array of cyto- and chemokines. This observation falls in line with a preceding study from Lichtenauer et al. where ATG conferred beneficial effects secondary to induced paracrine factors in an experimental model of myocardial infarction (Lichtenauer *et al.*, 2012). The induced cytokine profile shows overlap consisted with our findings.

In vitro assays confirmed that the upregulation of PD-L1 on monocytes is mediated by paracrine factors released in ATG-treated blood given that exposure of purified monocytes to conditioned medium from ATG-treated samples recapitulated the PD-L1 increase whereas direct stimulation with ATG did not achieve this effect. Amongst the induced cytokines we identified a central role of IFN-γ for induction of PD-L1. IFN-γ- mediated induction of PD-L1 is well-characterized as an evasion mechanism in tumor immunobiology (Butte *et al*, 2007; Chen & Han, 2015; Dong *et al*, 2002). Although generally considered as a proinflammatory cytokine, immunosuppressive mechanism mediated by IFN-γ relay back to its PD-L1

inducing properties (Garcia-Diaz *et al*, 2017; Rožman & Švajger, 2018). JAK-STAT signaling secondary to IFN-γ induces PD-L1 expression on tumor cells and increases resistance to NK-cell mediated cytotoxicity (Bellucci *et al*, 2015). These forms of immunological resistance by dampened susceptibility to cytotoxic lymphocytes have been reported for various tumor types (Gao *et al*, 2018; Knopf *et al*, 2023). Furthermore, host immune cell functions including co-stimulatory inhibition are exploited by tumor-specific microenvironment associated factors to enhance tumor resistance (Lau *et al*, 2017). Mechanism of T-cell modulation via co-inhibitory PD-1/PD-L1 are central mechanism in tumor evasion strategies and identification of this causality shifted momentum of therapeutic strategies in oncologic settings towards immune checkpoint inhibition (Butterfield & Najjar, 2024; Carlino *et al*, 2021). Blockade of immunoregulatory checkpoints (e.g., anti-PD-1 or anti-PD-L1 antibodies) targets PD-1/PD-L1 signaling to enhance T cell activity against tumors (Iwai *et al*, 2005; Zhou *et al*, 2010). While cumulative evidence suggest improvement for patients eligible for these therapies the exacerbated immunological reactions are often not limited to malignant cells but can lead to autoreactive damages on healthy tissues, leading to immune-related adverse events (Ramos-Casals *et al*, 2020). Indeed, checkpoint inhibitors often precipitate autoimmune toxicities including – but not limited to - myocarditis (Johnson *et al*, 2016), colitis (Beck *et al*, 2006) and hepatitis (Kleiner & Berman, 2012) in a subset of patients. Remarkably, ATG has been used as a rescue therapy for severe checkpoint-inhibitor induced autoimmunity. Case reports document that ATG can reverse fulminant myocarditis and refractory hepatitis caused by PD-1/CTLA-4 blockade (Jain *et al*, 2018). Taken together, while known T cell-depleting actions of ATG reduce the overshooting immune response our findings add an additional mechanistic layer by suggesting that checkpoint inhibitor related toxicity might be reversed by re-engaging the PD-1 pathway via PD-L1 induction on monocytes and other cell types.

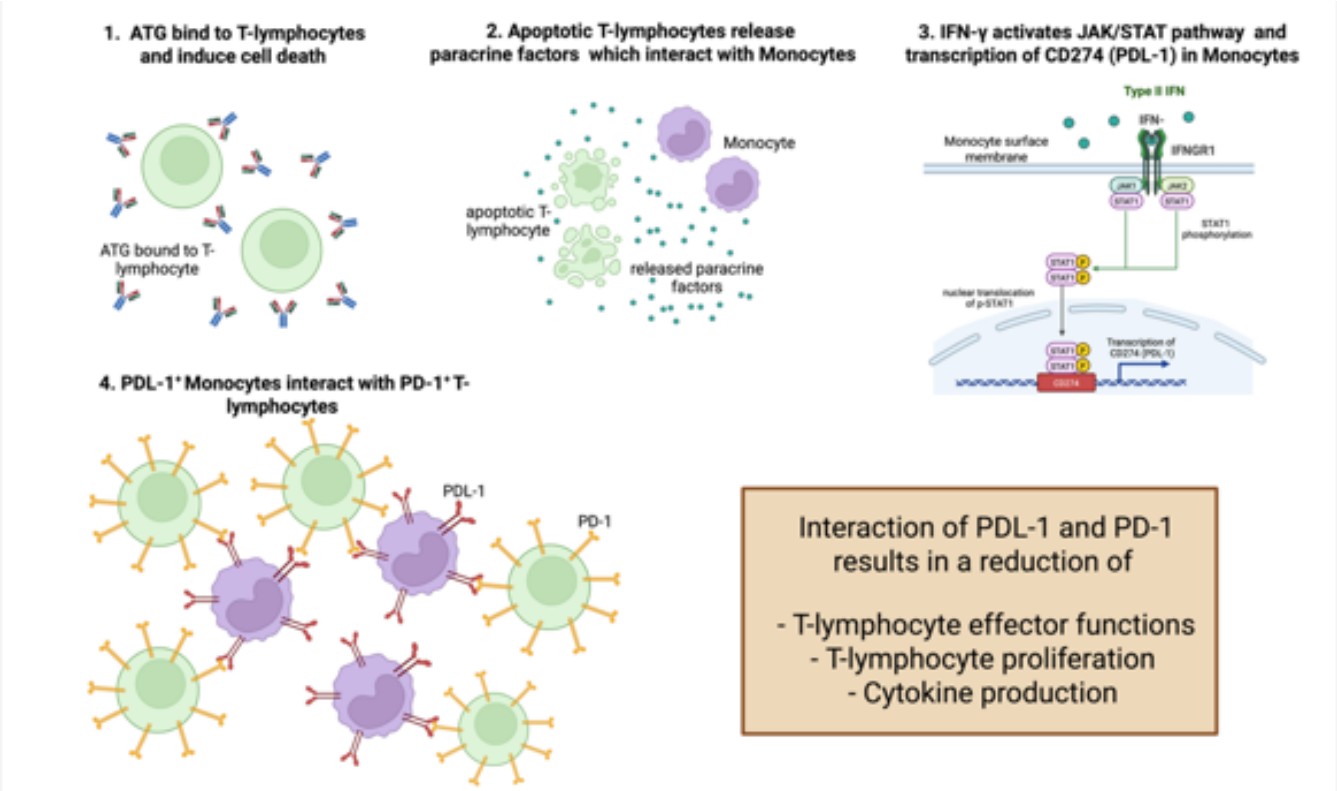

Figure 7. Graphical Summary: Paracrine induction of PD-L1 on monocytes to modulate T cell effector functions. (1) Anti-thymocyte globulin (ATG) binds surface antigens on T-lymphocytes and triggers T cell depletion. (2) Apoptotic T cells release paracrine mediators including IFN-γ. (3) IFN-γ engages the IFN-γ receptor on monocytes to activate JAK/STAT pathways to promote transcription of CD274 which translates to increased PD-L1 surface expression. (4) PD-L1$^+$ monocytes interact with PD-1$^+$ T-lymphocytes to inhibit T-cell effector functions such as T-lymphocyte proliferation and release of cytolytic cytokines. Created in https://BioRender.com

The PD-1 receptor on T cells and its ligand PD-L1 are universally recognized as critical modulators of peripheral tolerance with major implications for transplant acceptance (Francisco *et al*, 2010; Ozkaynak *et al*, 2002). Clinical use of ATG is in large parts reserved for hematologic diseases and transplantation medicine (Clayton *et al*, 2019; Gaber *et al.*, 2010; Luo *et al*, 2019). The induction of tolerogenic signals through PD-1/PD-L1 interaction in response to ATG and secondary mediators aligns with the functional and mechanistic aspects of immunosuppression. We demonstrated that PD-L1+ monocytes induced by ATG-conditioned-medium potently inhibit CD8+ T cell effector functions such as proliferation and cytotoxic molecule release. Antagonization of the PD-1/PD-L1 interaction by addition of the PD-L1 specific blocking-antibody durvalumab to PD-L1+ monocytes prior to co-culture, reversed this inhibition underscoring that the PD-L1 upregulation on monocytes was directly

responsible for the suppressed T cell activity. Together, these findings unravel an additional mode of action for ATG in indirectly imposing T-cell checkpoint inhibition by co-opting monocytes to express PD-L1. Thereby ATG amplifies and supports the natural mechanisms of peripheral tolerance. Furthermore, induction of the PD1-/PD-L1 co-stimulatory pathways after stimulation with ATG and its relayed secondary signaling mechanisms aid explanation to clinical observations in transplantation medicine that could not fully account T cell depletion for the observed effects (Aschauer *et al*, 2022; Gurkan *et al*, 2010). Several studies in experimental models of solid organ transplantation have shown the detrimental effect of PD-1 or PD-L1 inactivity (Chamoto *et al*, 2023; Liang *et al*, 2003). The requirement for PD1/PD-L1 signaling in upholding graft tolerance was exemplified in a single antigen mismatch model of graft rejection (Koehn *et al*, 2008). Early T-cell infiltrations in murine kidney transplantation model were diminished in response to PD-1/PD-L1 interactions (Shim *et al*, 2020). Complementary to this reports, overexpression of PD-1 in T-cells promoted allograft tolerance in a fully MHC-mismatched model for cardiac transplantation (Borges *et al*, 2021). Gain of function for PD-1 overexpression led to improved cardiac graft acceptance and decreased rejection rate and severity in animal studies. This reported effect was depended on interactions with PD-L1 and furthermore associated with an ICOS-dependent regulatory mechanism (Borges *et al.*, 2021). Moreover, PD-L1 expression on non-immune cells in the graft, such as endothelial cells, correlates with protection from T cell-mediated injury in a section of heart transplant patients, as higher PD-L1 expression on graft endothelium showed fewer infiltrating CD8+ T cells in endomyocardial biopsies (Bracamonte-Baran *et al*, 2021). Moreover, experimental ablation of PD-L1 on graft endothelial cells led to precipitous rejection in affected mice (Bracamonte-Baran *et al.*, 2021). Other than immune and endothelial cells, renal tubular epithelial cells also displayed functional plasticity for PD-L1 induction after exposure to interferon (Starke *et al*, 2010). These in vitro findings were confirmed in biopsy specimen from human renal transplant recipients with acute vascular rejections (Starke *et al.*, 2010). The increased PD-L1 signature observed in these tissue samples underscores the significance of inducing co-inhibitory stimulation as a countermeasure to graft damage (Starke *et al.*, 2010). Together these findings support the idea that PD-1/PD-L1 interactions significantly protect grafts from autoimmune reactions. Our data indicate that ATG can exploit this effect by upregulating PD-L1 on circulating monocytes to subsequently migrate to lymphoid organs or grafts and interact with PD-1 on alloreactive T cells. This modulation of peripheral tolerance not only depletes T cells via ATG but also exerts a suppressive influence on remaining or newly emerging T cells through co-stimulatory inhibition. This hypothesis is supported by our observation that ATG's effect on PD-L1 is sustained even in the presence of concomitant immunosuppressants (e.g.,

corticosteroids) and could provide a lasting immunomodulatory signal to aid in prolonged peripheral tolerance.

Concludingly, ATG operates on a multifaceted immunomodulator level. Depletion eliminates a large portion of alloreactive T cells initially while the expansion of T_{regs} helps control the regeneration of the T cell pool. Additionally, PD-L1+ monocytes directly inhibit activated T cells that escape deletion or suppression. Our data thus add to the broad spectrum of how ATG achieves high efficacy immunosuppression of T-cell effector functions. Altogether, our mechanistic insights provide a rationale for favorable outcomes in clinical settings of immunosuppression mediated by ATG that go beyond T-cell depletion.

3.3 Clinical implications for transplantation medicine

The elucidation of these paracrine immunomodulatory mechanisms carries significant implications for clinical utility. Dissecting the temporal dynamics for PD-L1 induction in patients might aid in optimization of ATG therapy duration and combination with other immunosuppressive therapies to maximize its tolerogenic effect. In practice, ATG is reserved for high-immunological-risk transplant recipients and is administered as a multi-dose induction regimen immediately before and in the days after transplantation (Aschauer *et al.*, 2022).

A single dose exposure to ATG *ex vivo* produced sustained PD-L1 expression in monocytes. This raises questions about how long PD-L1–expressing monocytes might persist *in vivo* after ATG induction and whether sustained PD-L1 correlates with improved graft outcomes. Monitoring PD-L1 on circulating monocytes after ATG induction or alternatively detecting increased levels of soluble forms in blood or urine could reveal whether high PD-L1 correlates with favorable graft outcomes. In oncologic disease high levels of PD-L1 have been associated with worse outcomes and high levels of soluble forms of PD-L1 associated with increased risks for infection (Chen *et al*, 2017; Negrao *et al*, 2021). However, in the setting of transplantation, PD-L1 could be used as an additional biomarker to monitor either the overall degree of immunosuppression or response to ATG therapy when used as induction therapy or treatment of cellular rejections. Such a biomarker might enable personalized instead of strictly weight-based dosing and ATG could be titrated to achieve a target PD-L1^{+} monocyte level where immunosuppression is stable and infectious complications are kept low. These changes would naturally require correlation with clinical and histopathological responses.

Another important factor is the influence of concomitant immunosuppressants on ATG-induced PD-L1. Standard immunosuppressive regimens typically use a calcineurin inhibitor

(CNI, e.g., tacrolimus), an antiproliferative agent and glucocorticoids (Menon & Murphy, 2013). Glucocorticoids did not diminish ATG-induced PD-L1 upregulation, indicating steroid use does not interfere with this mechanism in the *ex vivo* model employed in our study. In contrast, CNI may attenuate PD-L1 induction by suppressing T-cell IL-2 production and thus reducing IFN-γ (Ahmed *et al*, 2001). ATG is administered peri-transplant, before CNI levels reach their targeted levels, providing a window for cytokine-driven PD-L1 induction. ATG-induced PD-L1 may promote early graft tolerance and help mitigate chronic T cell–mediated injury, potentially preserving long-term graft function. Interestingly, preclinical data from Dahlquist et al. revealed conserved expression of PD-L1 on moDCs co-cultured with tacrolimus (Dahlqvist *et al*, 2021). However, a clinical trial investigating PD-L1 induction in patients receiving tacrolimus with and without ATG is required to clarify the impact of CNI and other potential confounders.

3.4 Clinical implications for PBMCsec in regenerative medicine

PBMCsec stimulates regenerative factors from host cells in a multi-modal therapeutic approach. PBMCsec offers a single intervention that integrates the delivery of pro-angiogenic and cytoprotective stimuli along with the here firstly described de novo induction of endogenous anti-protease activity. Former preclinical models revealed therapeutic efficacy across many disease states ranging from promotion of wound healing in burns and skin injuries to reduction of infarction size in heart and brain ischemia (Beer *et al.*, 2016). Our findings add an additional mechanistic layer to the explanation of these broad effects. In myocardial infarction, early invasive recanalization with reperfusion saves tissue but also triggers influx of inflammatory cells and proteases that extend damage secondary to reperfusion injury (Welt *et al*, 2024). The induction of protease inhibitors and strengthening of endothelial barriers by PBMCsec could attenuate such reperfusion-associated injury. The induction of VEGFA and other angiogenic chemokines by monocytes would further aid in revascularizing of ischemic border zones supporting recovery of tissues at risk. Clinical studies are needed to address safety, optimal dosing, and timing of potential interventions incorporating PBMCsec. A potential setup might involve the peri-interventional use of PBMCsec in relation to coronary artery reperfusion to harness peak influx of when immune cells flood myocardium. Previous strategies included antagonization of IL-1-β (Abbate *et al*, 2022) and early use of beta-blockers following reperfusion (Pizarro *et al*, 2014). The mechanistic actions of direct PBMCsec mediated actions as wells as secondary effects by immunomodulation of infiltrating cells on-site could be exploited similarly to synergistically promote tissue-protection. Our ex vivo whole-blood data provide a proof-of-concept that even short-term exposure to PBMCsec reprograms immune cells within hours, which suits the

acute treatment paradigm. Beyond that, the complexity of PBMCsec's composition naturally poses challenges for regulatory approval – as a mixture of proteins, lipids, and vesicles with between-batch-consistency must be ensured. Previous studies reported on the fulfillment of essential requirement to ensure reproductivity of virologically and toxicologically cleared GMP-compliant PBMCsec (Laggner *et al*, 2020b; Wuschko *et al*, 2019). The ongoing expansion of mechanistic actions of PBMCsec by precise identification of affected pathways, such as SerpinB2/PAI-2 induction, can potentially be referenced to guide both quality control and application protocols for future use.

3.5 Paracrine immunomodulation as a therapeutic strategy

Our work on PBMCsec and ATG highlights the wide spectrum of therapeutic immunomodulation of the immune cells by targeting interactions secondary to paracrine factors rather than direct cell-cell interactions. Both interventions leverage paracrine signaling pathways – PBMCsec delivers a multitude of signals to promote a regenerative secretory profile while ATG incites a cytokine-driven regulatory feedback loop to promote co-stimulatory inhibition. These effects stand in contrasts with more traditional approaches that either add an external signal in the form of a single cytokine or drug that acts directly on pathogenic cells (Lecocq *et al*, 2019). For instance, inducing SerpinB2 via PBMCsec makes use of the body's own arsenal of anti-protease defenses, potentially achieving a high local concentration at sites of injury with spatiotemporal precision. Similarly, ATG's use of IFN-γ to upregulate PD-L1 co-opts an intrinsic immunological checkpoint that finely tunes T cell activity. Thereby, these therapies utilize inherent regulatory mechanism to exert their effects. There is growing recognition that single-cytokine therapies can fail due to redundancy in immune networks and evasion mechanisms (Jenkins *et al*, 2018). By contrast, a complex intervention as mediated by a multifactorial secretome or a polyclonal antibody can address multiple pathways at once which requires accounting for off-target effects. PBMCsecs broad action on angiogenesis, anti-inflammation, anti-protease are key elements for sustained tissue repair. Its successful application would validate the strategy of cell-free biologics that deliver a therapeutic milieu rather than a single agent. For ATG, our findings integrate it into the modern context of immune checkpoint modulation emphasizing a converse manner to immune-checkpoint inhibitors by enhancement of the inhibitory checkpoint signals to treat immune-mediated diseases (Guo *et al*, 2022). Pharmaceutical development invested largely in inhibiting PD-1/PD-L1 to enhance treatment options for various cancer types. However, recent publications have introduced bioengineering and advanced life science techniques aimed at the opposite approach in the context of autoimmune diseases, such as systemic lupus erythematosus (Guo *et al.*, 2022) and type I diabetes (Yoshihara *et al*, 2020).

In the landscape of immunomodulatory research, our results fall in line with the key theme of the innate immune system as a therapeutic target for achieving immunological balance (Szollosi & Mathias, 2020). Traditionally, adaptive immune cells have been the focus in both autoimmunity and transplant rejection (Li & Lan, 2023). Yet, therapies like PBMCsec and ATG underscore that modulating innate immune cells – in our case monocytes but certainly not excluding macrophages, dendritic cells, NK cells – can profoundly influence adaptive immunity. Monocytes and macrophages are not just bystanders or passive antigen presenters but more actively involved in shaping the tissue microenvironment through cytokines, growth factors, and expression of co-inhibitory or co-stimulatory ligands (Parihar *et al*, 2010). Depending on the underlying pathophysiological context, immunomodulation of monocyte to express PD-L1 or to secrete SerpinB2/PAI-2 can be exploited to promote tolerance and tissue regeneration, respectively. Thus, both treatments exemplify the therapeutic reprogramming of innate immunity a strategy gaining momentum in areas ranging from cancer to chronic auto-inflammatory diseases (Bart *et al*, 2021; Vizcaino Castro *et al*, 2024).

3.6 Future directions and conclusion

These novel insights into PBMCsec and ATG open several avenues for future research. A priority is to translate and validate our *ex vivo* findings *in vivo* and into clinically relevant settings. For PBMCsec, it will be important to determine whether the secondary wave of factors observed *ex vivo* is also induced in living organisms. Animal studies could be designed where PBMCsec is administered systemically, and the host's circulating immune cells are then profiled for gene expression and protein secretion at various time points. Detecting induced factors like SerpinB2 in the plasma of treated animals would offer confirming data that the mechanism is conserved in an *in vivo* setting. Encouragingly, previous animal studies hinted at this by showing alterations in gene expression in distant organs following treatment with PBMCsec with our data now providing an additional mechanistic basis (Mildner *et al.*, 2022). An extension to this time-course studies will be critical to evaluate the duration of the induced changes. Our *ex vivo* whole-blood incubation was set at 8 hours while in living organism the stimulus may persist shorter or longer depending on pharmacokinetic determinants such as metabolization, clearance and feedback regulation. Furthermore, the concept of a dampened wave driving the observed effects suggests a finite duration, while also making it conceivable that repeated dosing of PBMCsec could extend the therapeutic window. A potential strategy that can be assessed in future studies might incorporate a two-phased delivery with an initial bolus for priming followed by subsequent smaller doses to sustain the secondary mediator production at a

lower level and potentially trigger mechanisms that require frequent activation. Besides these areas, identification of the components causative for the observed effects in monocytes following treatment with PBMCsec is a crucial goal. It is noteworthy that γ-irradiation of PBMCs induces programmed cell death and releases immunologically active alarmins (Simader *et al*, 2019). The thorough identification and characterization of these could link PBMCsec's mode of action to the phenomenon of sterile inflammation that prompts regeneration and would serve as an extension of previous studies (Beer *et al*, 2017; Beer *et al.*, 2015). Follow-up studies might consider utilization of combinatory therapy with specific inhibitors during *ex vivo* PBMCsec stimulation. As such blockade of TLR4 or alternative PRRs might be used to map the transcriptional alteration that led to the monocyte gene signature we observed (Ciesielska *et al*, 2021). Such mechanistic dissection could uncover targetable factors to either enhance desirable outputs or reduce any potentially deleterious ones – i.e. pro-inflammatory stimuli if counterproductive.

For ATG, a pressing next step is to evaluate whether PD-L1 induction can be recapitulated in treated patients. This could be approached by prospectively collecting blood from patients pre- and post-ATG induction therapy and measuring PD-L1 surface expression on monocytes by flow cytometry in combination with cytokine profiling for IFN-γ. If patient monocytes mirror our *ex vivo* findings, one could then correlate PD-L1 levels with transplant outcomes (acute rejection episodes, graft function, incidence of infections). Such a study would clarify the clinical relevance of ATG-induced PD-L1 and clarify whether it simply correlates with immunosuppression or actively impacts better graft tolerance. Additional variables like concomitant immunosuppressive medications, patient's immune status and the impact on outcome data could be simultaneously investigated. In addition, longitudinal studies during the typical two-week period of ATG induction could assess PD-L1 dynamics on immune cells *in vivo*.

Combination therapies and broader disease applications also emerge as future considerations. Thereby, ATG-mediated induction of PD-L1 could harnessed in autoimmune diseases outside transplantation. There is precedent for this approach as a trial of ATG in new-onset type 1 diabetes showed transient preservation of β-cell function, presumably by dampening autoimmunity (Haller *et al*, 2019; Haller *et al*, 2018). Monitoring PD-1/PD-L1 in such trials could provide additional insights.

While there is long-standing experience on the safety profile of ATG with the main side effects including fever, serum sickness and lympho- and thrombocytopenia our findings suggest monitoring for possibly prolonged immune alterations (Gaber *et al.*, 2010). A prolonged PD-L1 induction might further contribute to late effects such as impaired vaccine

responses or susceptibility to infections – both holding high priority when encountering affected patience in clinical practice (Lum *et al*, 1987; Tenney *et al*, 2015).

In summary, our studies expand on two distinct therapies – a cell-derived secretome and an immunoglobulin preparation – that both operate by modulating immune cell crosstalk. PBMCsec and ATG display how paracrine effects and secondary mediators modulate immunological functions that can aid in tissue regeneration or augmentation of peripheral tolerance. The novelty of PBMCsec inducing a secondary wave of paracrine factors from the host's own immune cells and ATG inducing immune checkpoint signals on myeloid cells expands our understanding of their mechanisms and suggests that comparable effects might be at play in similar therapies. Future research and clinical translation will determine optimal applications of these insights.

CHAPTER FOUR: MATERIALS & METHODS

A detailed description of the materials and methods applied in this thesis is provided in the respective publications.

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Clinical training

07/2022	Division of Nephrology and Dialysis, Department of Internal Medicine III Medical University of Vienna
05/2019 – 07/2019 Clinical Tertial C	Division of Cardiology, Department of Internal Medicine II, Medical University of Vienna
03/2019 - 05/2019 Clinical Tertial C	6th Medical Department with Nephrology and Dialysis and Outpatient Clinic, Klinik Ottakring Vienna
01/2019-03/2019 Clinical Tertial A	Clinic for General Internal Medicine Gastroenterology, Hepatology, Infectiology and Pneumology, Katharinenhospital, Klinikum Stuttgart
11/2018- 01/2019 Clinical Tertial A	Interdisciplinary Emergency Department (Ayuk *et al.*), Katharinenhospital, Klinikum Stuttgart
08/2018 – 11/2018 Clinical Tertial B	Division of Trauma Surgery, Department of Orthopaedics and Trauma Surgery, Medical University of Vienna
07/2018 Famulatur	Anesthesiology, General Intensive Care Medicine and Pain Therapy, Medical University of Vienna

07/2016 Famulatur	Primary care, Interdisciplinary Emergency Department (Ayuk *et al.*), Katharinenhospital, Klinikum Stuttgart
08/2015 Famulatur	Division of Pulmonology, Department of Internal Medicine II, Medical University of Vienna

EDUCATION

10/2019	PhD studies in the "Vascular Biology" program, Medical University of Vienna Thesis: „ Inducible paracrine factors from peripheral blood mononuclear cells modulate immunological function” Supervisor: Univ.Prof. Dr. med univ. Hendrik Jan Ankersmit, MBA
09/2017- 06/2022	Member of the Applied Immunology Laboratory under the guidance of Univ.Prof. Dr. med univ. Hendrik Jan Ankersmit, MBA
10/2013-07/2019	Human medicine, Medical University of Vienna
02/2012-11/2012	Civilian service as a paramedic with the Austrian Red Cross
07/2011	Matura completed with distinction
2003 – 2011 Feldgasse,	Realgymnasium und Wirtschaftskundliches Realgymnasium Realgymnasium

TEACHING ACTIVITIES

10/2023 treatment in	Block 14 – Teaching seminar on diagnostics and kidney dysfunction, Medical University of Vienna
03/2023 Praktisches	Tutor „Electrolyte disorders“ Integriertes Klinsch - Propädeutikum (IKPD/OSCE), Teaching Center,

Medical
University of Vienna

03/2023 Fall-Basiertes-Lernen (FBL 1 &2); Medical University of Vienna

03/2022 – 07/2022 TS Applied Immunology and Tissue Regeneration SS 2022, Medical University of Vienna

03/2022 – 07/2022 JC Current Topics in Applied Immunology SS22, Medical University of Vienna

03/2018 – 06/2018 ECG Tutor Integriertes Klinsch - Praktisches Propädeutikum
(IKPD/OSCE), Teaching Center, Medical University of Vienna

03/2015 – 07/2017 Tutor for Organ Morphology I & II in the group of Dr. med. univ. Waltraut Wasicky, Center for Anatomy and Cell Biology, Medical University of Vienna

AWARDS AND SCHOLARSHIPS

2019 Scholarship for outstanding academic achievements, Medical University of Vienna

2017 Scientific scholarship for students, Medical University of Vienna

2017 Scholarship for outstanding academic achievements, Medical University of Vienna

RESEARCH FOCUS

Immunobiology
- In vitro T-cell activation
- Interaction of myeloid and lymphoid immune cell subsets with a focus on regulatory co
receptors and their influence on immunosuppression

Transcriptomics
- Bulk-RNA sequencing
- Single-cell-RNA sequencing

Vascular biology
- endothelial dysfunction, interaction of endothelial cells with components of innate and adaptive immunity

LANGUAGE SKILLS

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FIRST AUTHORSHIP:

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