Diplomarbeit

Cytokine secretion level of peripheral blood mononuclear cells (PBMCs) after exposure to irradiated PBMCs secretome

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Inhaltsverzeichnis

1. Abstract

Background: Cell-based therapies have been tested in modern regenerative medicine to either replace or regenerate human cells, tissues, or organs and restore normal function. A growing body of evidence indicates that secreted paracrine factors exert beneficial biological effects that promote tissue regeneration.

The cell secretome is made up of diverse components which include a variety of proteins, lipids, and extracellular vesicles. Peripheral blood mononuclear cells (PBMCs), are capable of releasing significant amounts of biologically active paracrine factors modulating beneficial regenerative effects. The apoptotic PBMC secretome has been successfully used pre-clinically for the treatment of acute myocardial infarction, chronic heart failure, spinal cord injury, stroke and for augmented wound healing. The exerted effects are attributed to the secreted paracrine factors.

Methods: Naïve PBMCs were purified from heparinized blood samples and co-incubated with either resuspended GMP-APOSEC or a resuspension of lyophilized CellGRO at different concentrations. One panel of untreated cells served as control group. After 24h cells were removed from cell culture and the supernatants were obtained. Cytokine secretion levels were evaluated using Enzyme linked immunosorbent assay (Elisa) for selected lead-cytokines (Interleukin-8, BDNF, VEGF, ENA-78, and Gro-α, IL-1 - RA). Adding to that we investigated at which time points secretion levels were highest. Furthermore, cells were incubated under hypoxic conditions for a set time interval and thereafter the secretome was analyzed for alteration in cytokine secretion for each subgroup.

Results: Addition of the tested compounds at different concentration resulted in changed levels of cytokine secretion. The release of cytokines was augmented in the APOSEC treated cells and increased over time. The samples that had been moved to the hypoxia chamber and received GMP-APOSEC had higher values of cytokine secretion compared to the other two groups.

Conclusion: APOSEC seems to exert effects on PBMCs in which the release of certain growth factors, chemokines and interleukins is altered. Exposing the cells to hypoxic conditions resulted in a decrease of measured cytokine levels. However, for the cells that were treated with APOSEC we registered a reduced inhibition of their secretory ability. Further investigation will be needed to understand the complex interactions and functions the released cell - secretome has on viable PBMCs as well as other cells and tissues.

2. Zusammenfassung

Hintergrund: Zell - sowie sekretombasierte Therapien stellen die beiden bedeutenden Säulen der regenerativen Medizin dar. In präklinischen Studien konnte gezeigt werden, dass durch die Ausschüttung parakriner Faktoren biologische Prozesse bewirkt werden, welche die Regeneration von betroffenen Geweben begünstigt. Das Zellsekretom besteht aus verschiedenen Komponenten, zu denen unter anderem Proteine, Lipide, und Extrazelluläre Vesikel gehören. Die mononukleären Zellen des peripheren Blutes (PBMCs), sind in der Lage im Rahmen des programmierten Zelltodes, signifikante Mengen biologisch aktiver parakriner Faktoren freizusetzen. Dadurch werden regenerative Effekte in unterschiedlichen Geweben induziert. Die positiven Eigenschafen des apoptotischen PBMC-Sekretoms wurden in einer Reihe von präklinischen Studien nachgewiesen, welche Tiermodelle zu Pathologien wie dem akuten Myokardinfarkt, der chronischen Herzinsuffizienz, der traumatischen Rückenmarksverletzungen, dem Schlaganfall sowie der Wundheilung umfassten.
Methoden: Naive PBMCs wurden aus Blutproben isoliert und entweder mit GMP-APOSEC, CellGRO oder als Kontrollprobe ohne vorherige Behandlung kultiviert. Nach 24 Stunden wurden die Zellen aus der Zellkultur entfernt und die zellfreien Überstände mittels Zentrifugation gewonnen. Mittels Enzyme Linked Immunosorbent Assay (Elisa) bestimmten wir die freigesetzten Zytokinkonzentrationen von Interleukin-8, BDNF, VEGF, ENA-78 und Gro-α, IL1RA. Darüber hinaus wurden die Zellen unter hypoxischen Bedingungen für ein festgelegtes Zeitintervall (1h) inkubiert. Die Gabe der Präparate erfolgte in einem Versuchsansatz vor und im zweiten unmittelbar nachdem die Zellen in die Hypoxie-Kammer gebracht wurden. Aus den resultierenden Überständen bestimmten wir die Zytokinkonzentrationen.
Ergebnis: Unterschiedliche Konzentrationen von APOSEC verursachen unterschiedliche Sekretionsmengen durch die PBMCs. Die Sekretion nimmt über die Zeit hinweg zu. Unter hypoxischen Bedingungen nimmt die Menge an freigesetzten bioaktiven Substanzen ab. Auf jene PBMCs welche mit APOSEC behandelt worden waren hatte die Hypoxie eine geringere Auswirkung hinsichtlich Hemmung der Zytokinfreisetzung.
Schlussfolgerung: APOSEC beeinflusst die Freisetzung von parakrinen Faktoren aus den PBMCs. Zellen, welche mit APOSEC behandelt wurden, wiesen eine verminderte Hemmung ihrer Sekretionsfähigkeit unter Hypoxie auf. Es sind weitere Untersuchungen erforderlich, um die komplexen Wechselwirkungen und Funktionen, welche das Zellsekretom von PBMCs auf naive PBMCs und andere Gewebe ausübt, genauer zu verstehen.

3. Introduction

3.1 Regenerative Medicine

Replacement and regeneration of diseased tissues and whole organs incapable of performing this act autonomously is the main issue tackled by regenerative medicine. (1) Various approaches are employed for achieving this tasking endeavor. The strategies used in regenerative medicine focus on structural and functional reconstitution using cell based therapy, biomaterials - also referred to as scaffolds - and a combination of both. (1–3)
The concept of treating diseased patients with injected cell suspensions can be traced back to the Swiss physician Paul Niehans (1882 - 1971). He obtained organs from fetal and young animals and dissolved them in Ringer´s solution. The cells he derived by this procedure were then utilized for treatment of the corresponding tissues and organs in his patients. Niehans applied these xenogeneic cell suspensions in patients suffering from a variety of diseases ranging from neurological diseases, endocrine dysfunctions, heart failure, kidney disease to osteoporosis and various others. Based on his observations he argued that cell therapy was the way to go. The used xenogeneic cells did not evoke adverse effects but rather improved health in many of his patients. (1–3)
However the basic immunological knowledge has evolved enormously since then making it highly questionable that no adverse effects or rejection reactions occurred.(4)

3.1.1 Autologous vs Allogeneic cell therapy

Since these findings, a variety of different human cell types have been identified as source for cell-based therapy. (5) The cells used are either autologous, meaning they are derived from and, after the crucial step of ex vivo cultivation, transferred back to the same person, or allogeneic. In this case a donor provides cells for transfer to a recipient. (6)
For long-term repair of structural deficits such as skin replacement after severe burns or articular cartilage defects, autologous cell therapy has shown striking results. The applications of cultured autologous keratinocytes and cultured autologous articular chondrocytes were the first successful medical interventions of human autologous cell therapy. (6–8) In both settings the cells, isolated from patients' own tissues, changed their biological activities and natural morphology after removal from their physiological surroundings. Instead they were exposed to specific feeder layers augmenting their proliferative rate and influencing their secretory

pattern. This process of alteration in a mature cell from a differentiated state into another is termed dedifferentiation.
What was even more astounding, was the fact that after re-transplantation back to the patient, the acquired grafts re-differentiated towards their original state prior to extraction. The ability of cells to dedifferentiate, proliferate and then re-differentiate once they are transferred back to their physiological environment paved the way for long-term efficacy of autologous cell therapy. (8–10)

For indications where addition of biologically active cells facilitates stimulation of residual host tissue and restores it's innate function, allogeneic cell therapy holds more benefits than autologous cell therapy. (1,6) One of the major obstacles of allogeneic cells is their likeliness of being immunologically rejected by the recipient in the long run. This is an issue not present, when utilizing autologous cells. (11) However, even autologous cell therapy is not flawless. The huge expenses of harvesting and the expenditure for processing the patient's own tissues represent challenging hurdles to overcome. After all, the steps involve isolation, increase of the cell number and application to the patient and take up several weeks. For allogeneic cell therapy this does not apply to this extent. Cells that were obtained beforehand from one healthy volunteer can be distributed to a number of patients, which not only leads to cost reduction but also simplifies quality control and manufacturing procedures. (6)

3.1.2 Stem cells - a promising new frontier

As the concept of cell therapy evolved, it was only a matter of time for stem cells to attract more and more interest. (12) The most appealing traits for the involvement of stem cells are their ability for self - renewal and the capacity to differentiate into several adult cell types. (13) Among all types of stem cells, mesenchymal stem cells (MSCs) have sparked the most interest. They are non-hematopoietic stem cells derived from the bone-marrow, although numerous other tissues have been identified to contain MSCs and are capable of differentiating into a whole range of tissues including bone, cartilage, cardiac muscle and many more. (14,15) What makes them outstanding is that they have been demonstrated to lack immunological rejection reaction in hosts after allogeneic injection. (16,17)

Cardiac and vascular tissue regeneration have emerged as leading interests. (18)
Cardiac function is highly dependent on maintenance of cardiomyocyte homeostasis. In the event of acute myocardial infarction (AMI) oxygen supply to the myocardium is reduced, due to an occlusion in one or more coronary arteries.
Consequently, the fraction of cardiac tissue, that is reliant on nutrients and oxygen supply by the obstructed vessel, deceases and consequently after a series of events involving necrosis, inflammation and fibroblast proliferation scar formation takes place. The loss of cardiomyocytes leaves the remaining healthy myocardium at strain, which subsequently leads to congestive heart failure. (19) The innate capacity of human myocardium for regeneration is by no means sufficient to adequately compensate for the damage provoked by a catastrophic event such as AMI. (20) The discovery of resident cardiac stem cells (CSCs) overturned the long-lasting dogma that the heart was terminally differentiated and incapable of self-renewal. (21,22) For isolation and identification of this subpopulation of tissue specific stem cells, different cell membrane markers have been proposed, the most established ones being c-kit and stem cell antigen 1 (Sca1). (20,23,24)
Beltrami et. al. demonstrated the engraftment of c-kit+ cells in an animal model of myocardial infarction. They isolated cells from allogeneic female Fischer rats. The route of delivery was intramuscular injection into the region adjacent to the infarcted myocardium of the examined rats. Results from myocardial regeneration assays showed that c-kit+ cells regenerated cardiomyocytes and microvasculature not by fusion with preexisting cardiomyocytes, but through induction of proliferation. (23) However, human myocardium contains a rather small proportion of resident cardiac stem cells expressing the cell surface markers c-kit and Sca-1. (25)
Therefore, when other pre-clinical studies showed the presence of c-kit+ side-populations, which represent stem cells that do not originate from cardiac tissue and are capable of homing into cardiac tissue, a new opportunity for treatment of AMI had raised interest. (26–28)
Among others, it was the research group of Orlic et. al. specifically, that demonstrated new formation of cardiomyocytes, endothelial cells and smooth muscle cells after local transplantation of c-kit+ bone-marrow-derived cells in a murine model of myocardial infarction. (27) Taking these preclinical sets of data into consideration, the mode of action in which injected stem cells ameliorate cardiomyocyte regeneration was believed to result from their incorporation and transdifferentiation into cardiac tissue. (26,27) However translation from these preclinical findings to actual clinical settings did not progress as smoothly as anticipated. In a recently published meta - analysis by Gyöngyösi et. al. evaluating the effect

of intracoronary stem cell therapy after AMI they concluded that both clinical and functional parameters did not show significant improvement. They also put emphasis on the controversies present in cardiac cell-based regenerative studies. (29,30) One of the major issues that flaws the idea of cardiac repair via the route of stem cell engraftment is that the number of cells detectable in the tissue is, due to poor survivability, far too low to convincingly be accounted for the beneficial effect. (31)

3.2 Stem cells - a systematic overview

At the turn of the 20th century German scientist Ernst Haeckel (1834 - 1919) merged concepts of phylogenesis and ontogenesis to coin the term Stammzelle (stem cell). Later on, Theodor Boveri (1862 - 1915) and Valentin Haecker (1864 - 1927) established definite criteria regarding stem cells. Accordingly, for a cell to be considered a stem cell it needs to be capable of self-renewal and differentiation into somatic (adult) cells(32,33) The ability for one cell to differentiate into a vast number of different cell types (pluripotency) is the cornerstone of the immense interest in application of stem cells in regenerative medicine. (32) Many diseases such as heart failure, insulin - dependent diabetes, stroke, hematological disorders, neurodegenerative disorders such as Alzheimer's and Parkinson's disease, spinal cord injury, and kidney failure result from the loss of one or more critical populations of cells that the body is unable to replace.(32,34) Having the ability to generate clinically relevant numbers of defined cell populations provides the basic elements for tissue repair and regeneration. Therefore, translation of stem cell research into clinical therapy has become a tempting option in many fields. (35,36)

Based on the source of origin distinction between embryonic stem cells (ESCs) and adult stem cells (ASCs) can be made. (33) ASCs include tissue specific stem cells and bone-marrow derived stem cells (BMCs). (36)
The stem cell types representative of BMCs are hematopoietic stem cells (HSCs), mesenchymal stem cells (MSCs), as well as endothelial progenitor cells (EPCs). (36) Each of these entities have different properties and regenerative capabilities for tissue repair. (32,36)

3.2.1 Embryonic stem Cells (ESCs)

Embryonic stem cells are derived from early embryos. After in vitro fertilization, cells are cultured to the blastocyst state which arises around the fifth day of developmental stage. During this phase a group of cells named the inner cell mass (ICM), which eventually form all three germ layers - Ectoderm, Mesoderm, Endoderm - responsible for tissue development in the embryo, can be discriminated from an outer layer of cells referred to as the trophoblast. (37,38) At this early stage the cells of the ICM are still undifferentiated and have not yet

committed to become a specific cell type. (39,40) Originally, human ESC lines were derived using an immune-surgical method. During this procedure the blastocyst is briefly exposed to animal - antibodies directed against human cells combined with complement activation by guinea pig serum. In this process the outer cell layer gets dissolved. The antibodies are then rinsed and removed leaving only the intact ICM behind which in further course is utilized for cultivation and extensive research. (38)

However, this method for ICM preparation has its drawbacks. Exposure of the ICM to animal-derived products is not safe when human ESCs are used for transplantation. The possibility for pathogen transfer exists. As a consequence the patient's innate immune system can be triggered to a response bearing an increased risk of graft rejection.(38,41)
That is why methods such as laser-assisted blastocyst dissection and other microsurgical procedures for ICM retrieval with no such peril are preferable to immune-surgery. (41,42)
A common issue all of these approaches share is that destruction of early human embryo is obligatory for the extraction of human ESC from the ICM, raising serious ethical concerns. (38) Furthermore, ESCs cells are prone to immunological rejection by the host and are capable of teratoma formation when injected in vivo. (43)
While embryonic stem cells retain their ability to form all cell types in the body, adult stem cells are multipotent and can produce only a limited number of cell types. (39,44) Because of their plasticity and potentially unlimited capacity for self-renewal, embryonic stem cell therapies have been proposed for regenerative medicine and tissue replacement after injury or disease. (36)

3.2.2 Hematopoietic stem cells (HSCs)

HSCs are the foundation of hematopoiesis and represent the origin of the bloods ultimately mature effector cells including red blood cells, granulocytes, monocytes, dendritic cells, platelets, B- and T-lymphocytes and natural killer-cells (NK-cells). (45) A hematopoietic stem cell is a cell, isolated from the bone marrow or blood that can renew itself. Meaning that each time a HSC divides, one daughter cell retains the properties of a stem cell while the other differentiates along either the myeloid or lymphoid lineage. Additionally, mobilization out of the bone marrow into the bloodstream and the ability to perform programmed cell death are traits of a HSC. (36,46)

Hematopoiesis occurs initially during fetal development in blood islands by mesodermal cells of the yolk sac and the para-aortic mesenchyme. Throughout the gestation period other sites get involved. Around the third or fourth month of fetal development the formation of blood cells shifts towards the fetal liver. In the second trimester hematopoiesis predominantly takes place in the bone marrow and spleen. After birth the sites of blood cell formation become increasingly restricted to the bone marrow of flat bones including the sternum, ribs and iliac bone. (11,34,45,47)

During their differentiation process HSCs mature to hematopoietic progenitor cells (HPCs). (46) These cells include common lymphoid progenitors (CLPs) and common myeloid progenitors (CMPs). While the former account for the generation of B - and T- Lymphocytes as well as NK cells the latter is a precursor for red blood cells, platelets, granulocytes and monocytes. (48,49)

During maturation genetic regulators and a number of environmental factors such as cytokine and chemokine microenvironment play a key role in differentiation of HSCs. (47)

Hematopoietic regulatory cytokines are produced through both autocrine and paracrine mechanisms and in many cases are released by non-hematopoietic cells including bone marrow stroma and endothelium. (48) Cytokines involved in hematopoiesis are among others representatives of the Interleukin family (IL-7, IL-15, IL-3), stem cell factor (SCF), thrombopoietin (TPO), erythropoietin (EPO) and Granulocyte Colony Stimulating Factor (G-CSF). (34,48)

HSCs differ from CMPs or CLPs through expression of various cell surface markers and differences in gene expression signature. (49) The glycoprotein Cluster of differentiation 34 (CD34+) is the first marker characterized. Along with CD34+ others such as CD59+, Thy1/CD90 and c-Kit/CD117 have been identified as evidenced hematopoietic stem cell

markers. They are used to isolate HSCs for reconstitution of patients who have depleted hematologic formation capacity as a result of chemotherapy or disease. (45,49,50)

The long-term reconstitution potential of human HSCs has been directly shown in severe combined immunodeficiency disease - mice (SCID-mice) repopulating assays.
Genetically modified mice with SCID possess lymphocyte progenitors, but these cells are unable to survive until maturity. This results in a lack of B - and T - cells in the thymus and in the secondary lymphoid organs. (51) The treatment of SCID mice with CD34+ hematopoietic stem cells harvested from human bone marrow or thymus resulted in the formation of a functional human immune system within immune-deficient mice. The two established concepts are the SCID Thy/Liv and the SCID Bone models. Their design provides a useful tool to study the function of human HSCs and immune cells under physiologic and pathologic conditions in vivo. (52)

3.2.3 Endothelial Progenitor cells (EPCs)

The first description of EPCs can be traced back to 1997 when Asahara et. al. provided first evidence for circulating endothelial progenitor cells when they isolated putative EPCs from human peripheral blood. (53) Up until then the understanding was that postnatal neovascularization results from migration, proliferation and the remodeling of existing fully differentiated endothelial cells. (54) In their experiment they isolated cells from peripheral human blood and mapped them with antibodies binding to antigens characteristic for hematopoietic stem cells as well as angioblasts. (53,55) They then cultured these CD34+ and Vascular endothelial growth factor receptor 2+ (VEGFR-2+) cells on tissue culture plastic, fibronectin or type I collagen coated dishes. Attachment of the CD34+ cells on the fibronectin coated dishes was prompt, contrary to the one incubated with type I collagen. Furthermore, a morphological change towards spindle-shaped colonies was observed. Combined cultivation with CD34- from peripheral human blood led to the formation of cellular networks and tube-like structures on fibronectin-coated plates. The appearance and organization of these clusters resembled that of blood island-like cell clusters observed in dissociated quail epiblast culture, which gave rise to endothelial cells and vascular structures in vitro (53,55,56)

Additionally, to the findings in these in vitro experiments when CD34+, VEGFR-2+, CD34− or VEGFR-2− cells were injected into mice tail veins undergoing neovascularization due to

hind limb ischemia CD34+ and VEGFR-2+ cells, but rarely CD34− or VEGFR-2− cells, incorporate into the vasculature in a manner consistent with the emulating endothelial cells.(55,56)

Interestingly no cells could be traced in the unhindered limb indicating that EPCs are mobilized in response to ischemia or exogenous cytokines which further guide EPCs homing into sites of ischemic tissue, and thus participate in the repair and maintenance of vascular homeostasis. (53,56,57)
Improvement of neovascularization is a therapeutic option to rescue tissue from critical ischemia. (58) In animal models of myocardial infarction, the injection of ex vivo expanded EPCs or stem and progenitor cells significantly improved blood flow and cardiac function and reduced left ventricular scarring warranting the enormous potential these cells hold. (36,58)

3.2.4 Mesenchymal Stem Cells (MSCs)

Mesenchymal stem cells can be found in the bone marrow. Evidence for the presence of this cell type in the bone marrow was first provided by Alexander Friedenstein in 1976. (59) Along with his colleagues he placed whole bone marrow into plastic dishes. After incubation for 24-48 hours they discarded the non-adherent hematopoietic cells from the dishes, leaving diverging cells with different morphology behind. After cultivation time the cells became more homogenous in appearance resembling fibroblasts. They termed them colony forming units with fibroblastic properties (CFU-F). (59,60)

MSCs are non-hematopoietic stromal cells with multipotent properties capable of differentiating into and contributing to the regeneration of mesenchymal tissues such as bone, cartilage, muscle, ligament, tendon, and adipose tissue. (60) Apart from bone marrow MSCs have been isolated from various other sites such as adipose tissue, amniotic fluid, umbilical cord tissue (Wharton´s jelly and umbilical cord blood), periosteum and fetal tissues.(36,60) The manifoldness of sources to derive from explains the functional and phenotypical heterogeneity within MSCs. (61) The in vivo phenotype of bone marrow derived MSCs has been difficult to establish since the frequency of MSCs in the bone marrow aspirate lies at a low 0.001–0.01%. (60,61) Adherence to surface, proliferative nature along with the ability to form various cell types (multipotent) are characteristic traits of MSCs. (36) For purification surface molecules on cells are used to select a desired population or to remove unwanted cell

populations. Fluorescence-activated cell sorting (FACS) has been employed to for MSCs selection. (60,62)

Phenotypically, MSCs express a number of markers, none of which are specific to MSCs. However they can be distinguished from HSCs since they do not express cell surfaces molecules such as CD45, CD34, CD14, or CD11. (60) To add to these negative markers the low affinity nerve growth factor receptor (LNGFR), clustered as CD271 along with CD105 and CD73 are accepted as the most differentially expressed positive markers for MSCs distinction. (62)

3.3 Paracrine Factors – an important pillar

3.3.1 Cell secretome as facilitator of beneficial effects – a paradigm shift

One of the first groups that proposed the idea that the biological effects achieved by stem cell injection are not solely founded on cell- cell interactions but include the effects of secreted paracrine factors was the group of Gnecchi et. al. (63) The hypothesis for the involvement of paracrine factors resulted from their observations regarding stem cell engraftment in myocardium. In animal models, as well as in pre-clinical human studies the number of newly generated cardiac muscle cells and vascular cells after stem cell transplantation appeared too low to account for the cardiac repair and regeneration observed in the experiments. (64) In an animal model, using allogeneic rat MSCs, which were genetically modified to overexpress the prosurvival gene Akt (protein kinase B, PKB), they investigated whether cellular fusion between mesenchymal stem cells and recipient cardiomyocytes was the primary mechanism driving the repair processes after myocardial infarction. (65) Previously they had shown that injection of MSCs expressing Akt into the border zone surrounding infarcted myocardium in mice positively affected the healing process by reduction of necrosis, infiltration of inflammatory cells and scar formation. (65) In a subsequent experiment they used a X-gal staining to detect cellular fusion events. Furthermore they incorporated a method based on Cre/lox recombination recording LacZ expression, which in this approach only occurs after donor MSCs expressing Cre fuse with a recipient mice cardiomyocyte to determine MCSs engraftment and cell fusion. (65,66)
They observed that rather than transdifferentiating into cardiomyocytes the majority of the transplanted MSCs overexpressing Akt (Akt-MSCs) kept their original flat or spindle like morphology indicating, that the frequency of differentiation events is very low. However fusion events were observed albeit to an extremely low degree. (65)

Hence they and others proposed that secretion of soluble factors including several cytokines and growth factors exerts additional cytoprotective effects. (64) This idea is strongly supported by the results from various experimental studies showing that administration of cell free conditioned medium (CM) from adult stem cells is able to replicate the benefits following stem cell therapy. (58,67–70) There are several mechanisms for paracrine factors to affect damaged cells during events of major stress like myocardial infarction.
These include myocardial protection via cytoprotective molecules - cytokines from the interleukin family, growth factors and peptic hormones amongst others - subsequently

activating pathways promoting cell survival. These include the phosphatidylinositol 3-kinase (PI3K)/Akt pathway, protein kinase C pathway and others. (67) The terminal effect is activation of genes promoting survival, as well as transcription and translation of anti-apoptotic genes. (71)

Additionally, paracrine factors promote new vessel formation since they represent a source of proangiogenic factors such as vascular endothelial growth factor (VEGF), stromal cell derived factor 1 (SDF-1) and Angiopoietin-1 (Ang-1). (63,67,72)

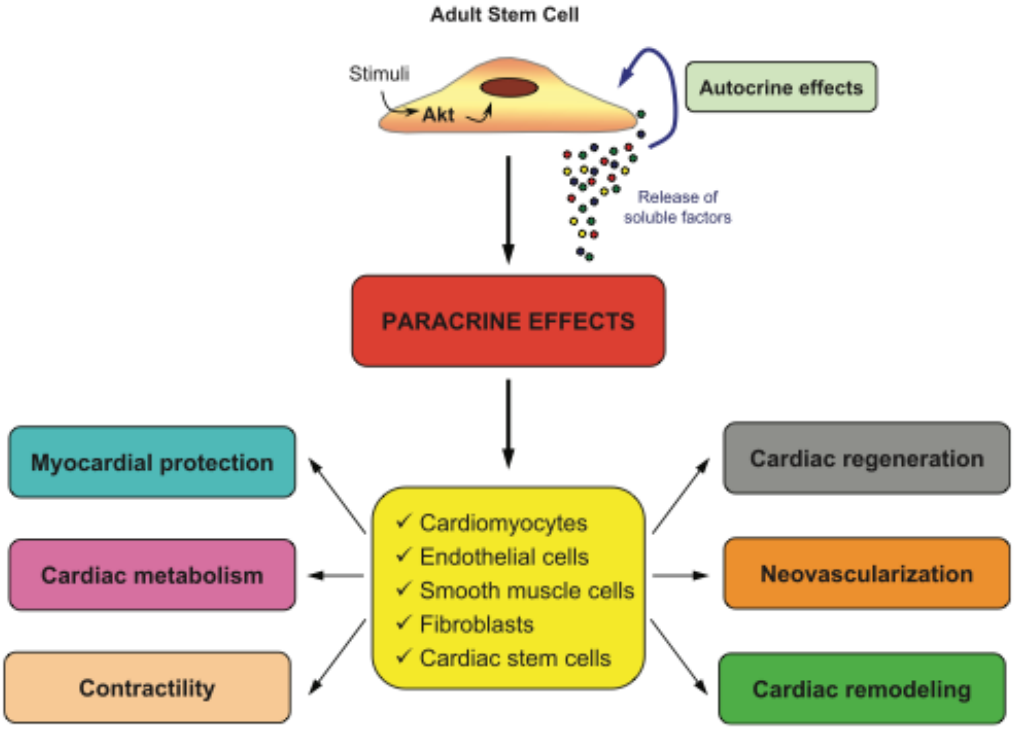

Figure 3.1 Paracrine secretome from Akt+ adult stem cells and its diverse effects in cardiac regenerative medicine. Soluble factors released from Akt+ adult stem cells facilitate autocrine effects. Adding to that, the secretome exerts paracrine effects on different cell types resulting in the modulataion of cell protective and regenerative mechanisms. This figure was adapted from Massimiliano Gnecchi, Zhiping Zhang, Aiguo Ni, et al. Paracrine Mechanisms in Adult Stem Cell Signaling and Therapy, Circulation Research, Volume 103, Issue 1, pages 1204 – 1219, https://doi.org/10.1161/CIRCRESAHA.108.176826 – last checked on the 8th of May at 17:30, license number: 4584270114895

3.3.2 Paracrine Effects of the stem cell secretome

The set of molecules released into the immediate space surrounding the cells is referred to as secretome. It includes soluble proteins, lipids and extracellular vesicles. (73,74) Furthermore, it is specific to individual cell types and tissues and changes in response to alterations in physiological homeostasis or pathological conditions. In recent years a great amount of research has been conducted to assess the effects of the stem cell secretome in highly challenging fields such as cardiovascular and neurodegenerative disease. (75) Several preclinical in vitro and in vivo studies have been performed in which the pro-angiogenetic, neuroprotective, anti-apoptotic and immunomodulatory properties of the released paracrine stem cell factors were demonstrated. (74,76,77)

Since stem cells can be derived from a vast array of different tissues, proteomic analysis of different populations of stem cells was conducted in order to determine differences in the released secretomes. (75,78) Especially for distinct populations of MSCs - including adipose tissue derived MSCs, human umbilical cord derived MSCs and bone marrow derived MSCs - important differences regarding amount of released VEGF, Hepatocyte growth factor (HGF), nerve growth factors (NGF) and other stem cell factors were detected in the investigated cell secretomes. (79,80) However these studies solely focused on investigating the proteomic content of the secretome which represents only a brief fraction of the many functional properties that constitute the cell secretome. (78)

In order to generate the stem cell secretome cells have to be obtained. Ex vivo cultivation of stem cells represents a tasking endeavor. Tissue stem cell numbers are very low making it very hard to isolate targeted cells. However aside from the stem cell secretome there have been other cell types identified with comparable traits but without the pitfalls regarding isolation, cultivation and secretome generation. (81,82)

3.3.3 Cell secretome release induced by paracrine factors – a further mode of action

The concept of understanding how stem cells exert their biological function developed from a cell-based theory towards the idea of a mode of action centered around paracrine factors and their influence on cell repair. (81) Apparent observations from many studies showed that not only transplanted stem cells are unable to sufficiently engraft and differentiate into targeted tissues in vivo but that during the process of cell application and transplantation great cell loss occurs. (64,83) Thus the initial understanding that the beneficial effects attributed to the MSCs were primarily due to local engraftment and transdifferentiation were overturned by

several groups that were able to demonstrate that the release of paracrine factors initiates cell repair mechanisms, pro-survival pathways as wells as angiogenesis.(73,84)
Adding to that, more and more evidence points to the fact, that the effects observed by the release of the cell secretome cannot be accounted to a specific subgroup of molecules but rather rely on the interaction of all components present in the secretome.(85)
Considering that we believe that by the release of paracrine factors a cell-to-cell signaling is commenced which in return facilitates additional paracrine factor release by the affected cells and augments the beneficial processes involving cytoprotection, angiogenesis and others.

3.4 Peripheral Blood Mononuclear Cells (PBMCs)

3.4.1 PBMCs subgroups

PBMCs include lymphocytes, monocytes, and dendritic cells. Lymphocytes can be further subdivided into T- Lymphocytes and B- Lymphocytes. (11,34)
The frequency of these populations varies across individuals. Lymphocytes are typically in the range of 70 – 90% of PBMCs, monocytes range from 10 – 30% of PBMCs while dendritic cells are rare. They make up approximately 1 – 2% of PBMCs. (11,86)
Cluster - of - Differentiation 3 (CD3) is a cell surface marker for lymphocytes.
The CD3+ compartment is composed of CD4+ (35-60%) and CD8+ T cells (15 – 40%). Both CD4+ and CD8+ T cells can be further subdivided into naive and the antigen-experienced, central memory, effector memory, and effector subtypes which exist in resting or activated states. They are the cellular mediators of acquired immunity. (11)
CD4+ T-cells are also referred to as helper T cells and can be further classified into various functional subtypes based on their expression patterns of specific cytokines, surface markers, or transcription factors. These include regulatory T-cells, TH1, TH2, and TH17 cells.
Circulating B cells include transitional, naive, and memory subtypes as well as plasma-blasts. They represent the humoral branch of the acquired immunity and exert their effect by antibody production in response to antigens. (11,86)
Circulating dendritic cells include plasmacytoid dendritic cells, as well as myeloid derived dendritic cells. Circulating monocytes have been described as either being typical monocytes or non-classical pro-inflammatory CD16+ monocytes. These comprise up to 10% of the monocytes in peripheral blood and have unique functions compared to typical monocytes. (11)

For human immune system studies peripherally drawn whole blood has to be divided into its components in order to remove red blood cells and other fractions from the targeted peripheral blood mononuclear cells (PBMCs). For this purpose, Ficoll Paque, a highly branched, high-mass, hydrophilic polysaccharide, is most commonly utilized. Whole blood is layered on the Ficoll gel and during centrifugation the blood components are separated according to their density gradient above or below the threshold of 1.077g/ml. (87)
Peripheral blood mononuclear cells (PBMCs) are the population of immune cells that remain at the less dense upper interface of the Ficoll layer. The cells can then be collected from this layer, which is also referred to as buffy coat.

Erythrocytes and polymorphonuclear cells (PMNs) including neutrophile granulocytes and eosinophile granulocytes are removed during this fractionation. (87)

3.4.2 Hypoxic preconditioning and its effects on PBMCs

All nucleated cells can sense and respond to a decrease in oxygen supply. (88)
At the cellular level, the majority of genes expressed after exposure to hypoxia are regulated by the hypoxia-inducible factor- 1(HIF-1). It constitutes of an α and β subunit forming a heterodimer. Under normoxic conditions, HIF-1α is rapidly degraded by proteasomes. However, under hypoxic conditions, HIF-1α does not undergo degradation. (89) It exerts transcription factor activity for various genes involved in angiogenesis , maturation of red blood cells, cell proliferation, and viability. (89,90)

3.4.3 Apoptotic peripheral blood mononuclear cells secretome (PBMCs)

When PBMCs are gamma - irradiated they enter apoptosis, a form of programmed cell death. (91,92) Throughout this process they put immunomodulatory effects in motion, support pathways that act via cytoprotection and regeneration and release growth signals that promote cell proliferation. (93,94) Once a cell enters the apoptotic pathway, a cascade of intracellular mechanisms gets in motion alerting and supporting nearby cells to enhance the chance for survival. The better part of these mechanisms can be accounted to actions of paracrine factors which get released during this process. (91,94) The apoptotic secretome (APOSEC) of PBMCs constitutes of a vast array of active agents. (94) More precisely, the components can be divided into different subgroups based on their molecular characteristics. (92,94) A distinction between proteins, extracellular vesicles (e.g., microparticles and exosomes), and lipids can be made. They have been shown to exert biological activity in vitro as well as in vivo experiments. (94)

The secretome of stressed PBMCs operates on many different levels, ranging from enhancement of angiogenesis, antimicrobial activity, reepithelization, augmented release of neurotrophic factors and many more. (94) Proteins and exosomes have been shown to be the two main active components that propagate pro-angiogenic gene expression in irradiated PBMCs secretome. (92) Cytokines (IL-1, IL-8, TGF-β), chemokines (ENA-78, Gro-α) and growth factors (VEGF, BDNF) are constituent parts of the protein fraction and explain the

multifunctional mode of action to some degree. (82,92) Further investigations are needed to clarify which interactions between the many identified active agents in the PBMCs secretome are imperatively necessary for the diverse beneficial effects at hand. (94)

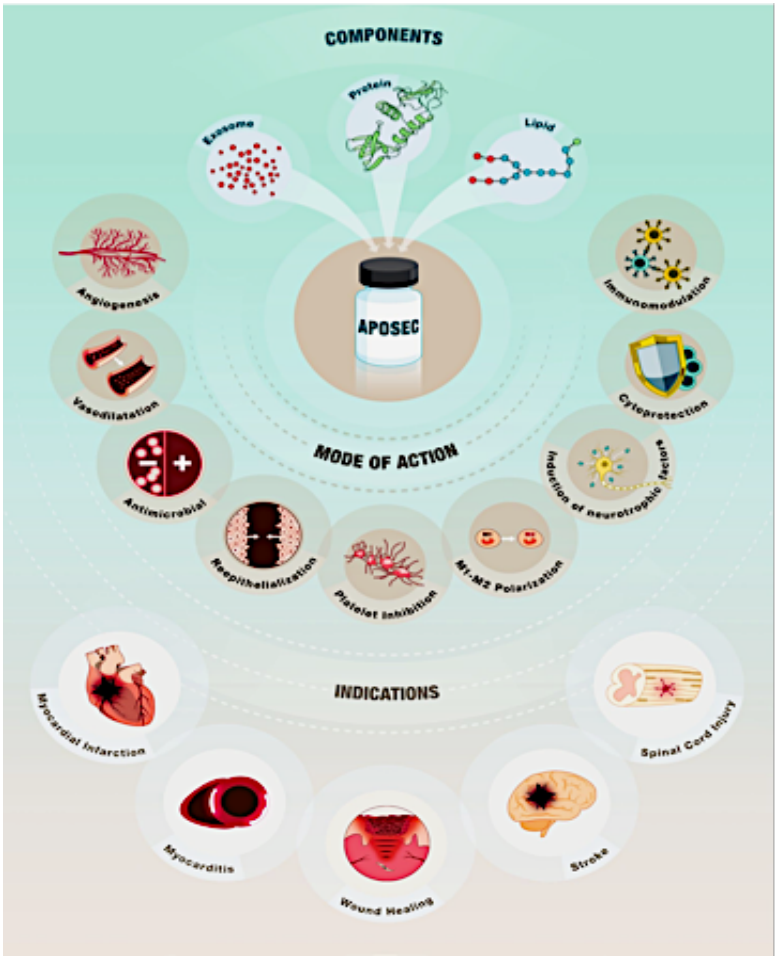

Figure 3.2 An overview over the broad field of applications for APOSEC. This Figure depicts the components present in APOSEC that have been identified to act out the biological effects as well as various modes of action which have been demonstrated in previously conducted preclinical trials. Furthermore, this figure includes an overview over some indications where application of APOSEC showed beneficial effect in preclinical trials. This figure was adapted from Beer et al. https://creativecommons.org/licenses/by/4.0/legalcode - last checked on the 8th of may 2019 at 16:00

3.4.4 Preclinical and clinical implementations of apoptotic PBMCs - secretome

The application of apoptotic PBMCs secretome has shifted more and more into consideration for cell free regenerative therapies. (94) Several preclinical studies have shown the beneficial properties of the apoptotic secretome.(94)
Ankersmit and his research team were able to show that the secretome of gamma-irradiated PBMCs exerts anti- inflammatory effects and ameliorates myocardial damage in a rat model of acute myocardial infarction (AMI). (95)

Lichtenauer et al. demonstrated the impressive potential of intravenously applied irradiated PBMCs secretome in a porcine large animal model. (82) Through balloon dilation the left anterior descending artery (LAD) was occluded for a total time of 90 minutes. After the set time, the occlusion was removed and reperfusion was established, mimicking a closed chest reperfusion model in acute myocardial infarction (AMI). (82)
Forty minutes after the start of the LAD occlusion supernatant obtained from irradiated apoptotic porcine PBMCs was administered intravenously during the timespan of 25 min. After a total of 90 minutes of vessel occlusion, the balloon was deflated, establishing reperfusion. Serum free cell culture medium served as control to the irradiated apoptotic porcine PBMC secretome. (82)
The results were astounding, considering that pigs receiving apoptotic cell secretome had a better ejection fraction (57.0 % versus 40.5 %), improved cardiac output (4.0 l/min versus 2.4 l/min), and registered a reduction of infarct area size (12.6 % versus 6.9 %) compared to controls. (82,94)

In a rodent spinal cord injury model by Haider et al. the application of human PBMC secretome resulted in an attenuation of secondary damage compared to control treated animals. (96) The observed neurological improvement of the rats - evaluated with the BBB-score - came along with a visible reduction in lesion volume of histological samples as well as diminished axonal damage. (96) Furthermore, the animals receiving irradiated PBMCs secretome showed a significantly higher CD68+ cells count in the dorsal column 3 days after spinal cord injury compared to control animals.
This marker is specific for various cells of the macrophage lineage that are important facilitators of the immune response in injuries to the nervous system. This observation

advocates that neuroprotective properties due to immunological mechanism accelerate attenuation of secondary damage after spinal cord injury. (94,96)

The neuroprotective properties cover more grounds than solely injuries to the spinal cord. (94) Additionally, to this observation in a separate preclinical study performed by Altmann et al. the secretome of apoptotic PBMCs ameliorated neurological damage in rats with induced focal ischemia. (97) In 29 animals permanent occlusion of the middle cerebral artery to the right hemisphere was established by insertion of a 3 cm coated monofilament with a thickened tip via the external carotid artery and advanced until in place of the targeted vessel. (97) These animals received re-suspended lyophilized human apoptotic PBMCs secretome intraperitoneally. Administration of the compound was performed forty minutes and 24h after focal ischemia was induced. (97) In comparison to control medium the animals treated with the secretome of human apoptotic PBMCs improved over time and showed reduction in neurological symptoms when evaluated. (97)

Furthermore, the angiogenetic and neurotrophic properties of the apoptotic human PBMCs secretome were demonstrated by several groups by performing various assays including an aortic ring sprouting assay as well as a neural sprouting assay in human primary neurons. Observations of new vessel formation and significant increase in the length of newly sprouting neurons respectively are, among others, attributable to the presence of VEGF and BDNF in the secretome and make a case for the vast beneficial activities this compound contains. (96–98)

In addition to that, the apoptotic PBMC secretome has been shown to exert antimicrobial activity through the release of peptides such as cethelicidin and calprotectin. An inhibitory effect on growth both in gram positive and gram negative bacteria was described. (99) After depicting enhanced wound healing in vivo and in vitro including accelerated re-epithelialization and neo-angiogenesis (100) coupled with upregulation of pro-survival pathways promoting cytoprotection and augmented proliferation in skin tissue (101) the apoptotic PBMCs secretome was approved for a Phase I clinical trial to investigate its safety and tolerability when topically administered in human intact skin, as well as on open wound area. (102) This was the first clinical prospective study using autologous apoptotic PBMC secretome in humans. The secretome was shown to be safe and tolerable meeting the primary endpoint set in this study.

Species	Experimental model	Effects on disease	Application	Concentration at cultivation	PBMC source	Apoptotic stimulus
Rat	AMI	Reduced infarct size, improved functional parameters	i.v.	25×10^6	Syngen	γ-irradiation (60 Gy)
Pig	AMI		i.v.	25×10^6	Syngen	γ-irradiation (60 Gy)
Pig	AMI		i.v.	25×10^6	Syngen	γ-irradiation (60 Gy)
Mice	EAM	Resolution of acute inflammation	i.p.	25×10^6	Syngen	γ-irradiation (60 Gy)
Mice	Dermal wound	Improved wound healing	Topical	25×10^6	Syngen	γ-irradiation (60 Gy)
Pig	Chronic HF	Improved functional parameters	i.m.	25×10^6	Syngen	γ-irradiation (60 Gy)
Rat	Stroke	Reduced infarct size, improved neurological parameters	i.v.	25×10^6	Syngen/ human GMP viral cleared	γ-irradiation (60 Gy)
Rat	SCI	Reduced trauma size, improved neurological parameters	i.p.	25×10^6	Human GMP viral cleared	γ-irradiation (60 Gy)
Pig	Dermal wound	Improved wound healing	Topical	25×10^6	Human	γ-irradiation (60 Gy)
Pig	AMI	Reduced infarct size, improved functional parameters	i.v.	25×10^6	Syngen GMP viral cleared	γ-irradiation (60 Gy)
Human	Dermal wound	Safety and tolerability	Topical	25×10^6	Autologous GMP	γ-irradiation (60 Gy)

Figure 3.3 Preclinical application of APOSEC. Several preclinical studies in small as well as in large animal have been conducted in previous years. This graph gives an overview over the clinical pathologies including a number of animal models and a human safety and tolerability study.
The observed effects of APOSEC in each experimental setting are depicted. Furthermore, source of PBMCs acquisition, concentration of PBMCs at cultivation, the apoptotic stimulus for initiation of programmed cell death and the route of administration are shown. Adapted from Beer et al.

4. Aims of the study

The aim of the study is to determine, whether incubation with the secretome of irradiated peripheral blood mononuclear cells (PBMCs) leads to a change in the biological function and release of selected cytokines (IL-8, BDNF, VEGF, ENA-78, Gro-α, and IL1-RA) in non-irradiated cultivated PBMCs.
In addition, we want to investigate the influence hypoxic preconditioning has on PBMCs and whether concurrent incubation with APOSEC alters the secretion pattern of viable PBMCs.

Thus, we want to investigate, if the beneficial effects of APOSEC may be induced by the released paracrine factors within the cell secretome inducing the release of paracrine factor secretion in viable PBMCs. Therefore, cells from healthy volunteer blood donors were obtained and further processed in cell culture ex vivo.
This would add to the further understanding of the immunomodulatory properties APOSEC holds.

5. Methods

The approval for this study was granted by the ethics committee of the Medical University of Vienna, Austria (ECS number 1539/2017).

5.1 Experimental Methods

Peripheral blood mononuclear cells (PBMCs) were separated from heparinized blood samples by Ficoll Paque-density gradient centrifugation. These were acquired at the Department of Transfusion Medicine, General Hospital Vienna.
After separation cells were co-incubated with APOSEC, lyophilized CellGRO or left untreated for 24h. Additionally we investigated the effect of hypoxic preconditioning of the PBMCs and left them in a hypoxia chamber for 1h, 2h and 24h.
Then using ELISA, we analyzed the supernatants content, focusing on lead cytokines including VEGF, BDNF, ENA-78, Gro-α, MMP-9, IL-1RA and IL-8.

5.2 Ficoll-Separation of PBMCs

Peripheral blood mononuclear cells (PBMCs) were purified by Ficoll-Paque PLUS (GE Healthcare Bio-Sciences AB, Sweden) density gradient centrifugation. Anticoagulant-treated blood was diluted with balanced salt solution and then 25ml of this dilution were carefully layered over 15ml of Ficoll-Paque PLUS in a 50 ml centrifuge tube.
For this crucial step, a centrifugation protocol including 800g for a duration of 15 minutes and slow acceleration and slow deceleration was employed.
After centrifugation at room temperature lymphocytes, along with monocytes, NK cells and further mononuclear cells were harvested from the interface between the Ficoll-Paque PLUS and the plasma layer.

The obtained cells were then centrifuged twice with Hank`s balanced salt solution (ThermoFischer Scientific, Waltham Massachusetts USA) to wash the lymphocytes - settings at 1300 rounds per minute (rpm) for 2 minutes with maximum acceleration and deceleration. The resulting cell pellet was then re-suspended with CellGRO serum-free medium (Cell-Genix, Freiburg, Germany) and diluted to a concentration of 2,5 x 10^6 cells/ml.

5.3 Processing and cultivation of isolated PBMCs

The PBMCs obtained from each donor were treated with APOSEC (allogeneic APOSEC™, Lot number: 3999042) and lyophilized CellGRO. The Red Cross Blood Transfusion Service of the Red Cross of Upper Austria in Linz manufactured APOSEC according to recent Good manufacturing practice (GMP) Guidelines.
Blood donor acceptance criteria, procedures and documentation take place according to European and national regulations.

In order to properly look into our formulated question at hand we looked into several different study settings. For each one of those, blood samples of five donors were used. First, we tested a total of seven conditions within each donor. PBMCs of the donors were treated in triplicates. Cells were then diluted until a concentration of $3,3x10^6$ cells/ml was set – allowing a range from 3,4 - $3,6x\ 10^6$ cells/ml. Using Sysmex cell counter the number of cells in each donor stock was determined.
The lyophilized stock GMP-APOSEC was concentrated at $500x\ 10^6$ cells/ml. This stock was then diluted in 5ml NaCl which resulted in a concentration of $100x\ 10^6$ cells/ml.
In order to distinguish the best concentration for our stimulation, PBMCs were treated with GMP- APOSEC in varying concentrations of $25x10^6$ cells/ml; $12,5x10^6$ cells/ml and $2,5x10^6$ cells/ml of the GMP – APOSEC stock solution. The same applied for the lyophilized control medium CellGRO.
Addition of cell free treatment and control medium resulted in dilution of the original PBMC stock cell count. As a whole, cell free treatment and control medium together with a defined amount of 875µl of PBMC stock added up to 1ml in total, containing $2,5x10^6$ cells in each cell culture well. PBMCs which received neither GMP-APOSEC nor lyophilized CellGRO treatment would represent the seventh condition on the cell culture plate.
Therefore, for every donor a separate PBMC stock diluted at $2,5x10^6$ cell/ml was prepared. Each cell culture well contained a concentration of $2,5x10^6$ cells/ml. Additionally for reference values, the diluted APOSEC and CellGRO used for stimulation were concomitantly incubated for 24 hours on the same plate.

After incubation for 24 hours the contents of the cell culture wells were transferred to separate tubes. Using centrifugation - 2500 rpm for 9 minutes at room temperature - supernatants were separated from the cell pellet and once more transferred to Eppendorf tubes. The cell pellets in the Sarstedt tubes were conserved using 500 µl of peqGOLD TriFast™ which was utilized

for RNA extraction. The supernatants were frozen at -20°C usage for ELISA. The cell pellets were stored for RNA isolation at - 80°C until further processing. Analysis of the cell pellets was not further conducted and will be subject to following projects.

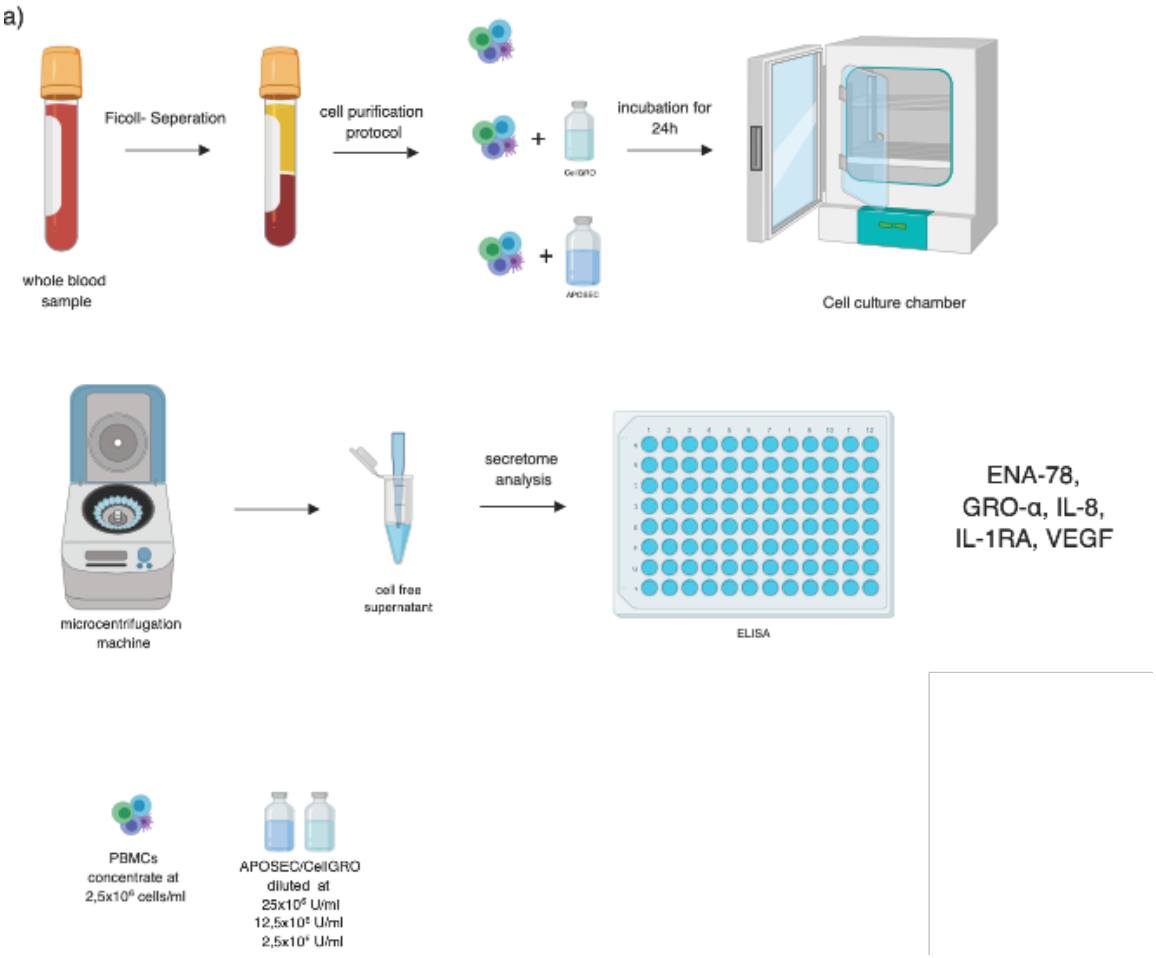

Figure 5.1. Preparation of cells and measurement for selected lead cytokines. PBMCs were isolated from heparinized blood samples and diluted to a concentration of 2,5x10x^6 cells/ml using CellGenix culture medium. PBMCs were then treated with either APOSEC, lyophilized CellGRO or left untreated. The treatmend compounds were applied in three different concentrations (25x10^6 cells/ml, 12,5x10^6 cells/ml, 2,5x10^6 cells/ml) and transferred to a cell incubator where they were present for 24h. Afterwards the cell free supernatants were obtained via centrifugation. We measured the concentrations of soluble factors – including ENA-78, Gro-α, IL-8, IL1-RA, and VEGF – using ELISA. This figure was created with BioRender.com, https://biorender.com last checked on 1st of May 2019

In addition to the processing described above, we wanted to assess whether the production of cytokines and growth factor occurred in a time dependent manner. To investigate this, we prepared the PBMCs as already mentioned but treated them solely with GMP-APOSEC or

lyophilized CellGRO at a concentration of 12,5x10^6 cells/ml. Untreated PBMCs with identical cell count served as control.

At defined time points which were set at 6 hours, 12 hours, 18 hours, and 24 hours we removed them from cell culture. Further processing was conducted as previously mentioned.

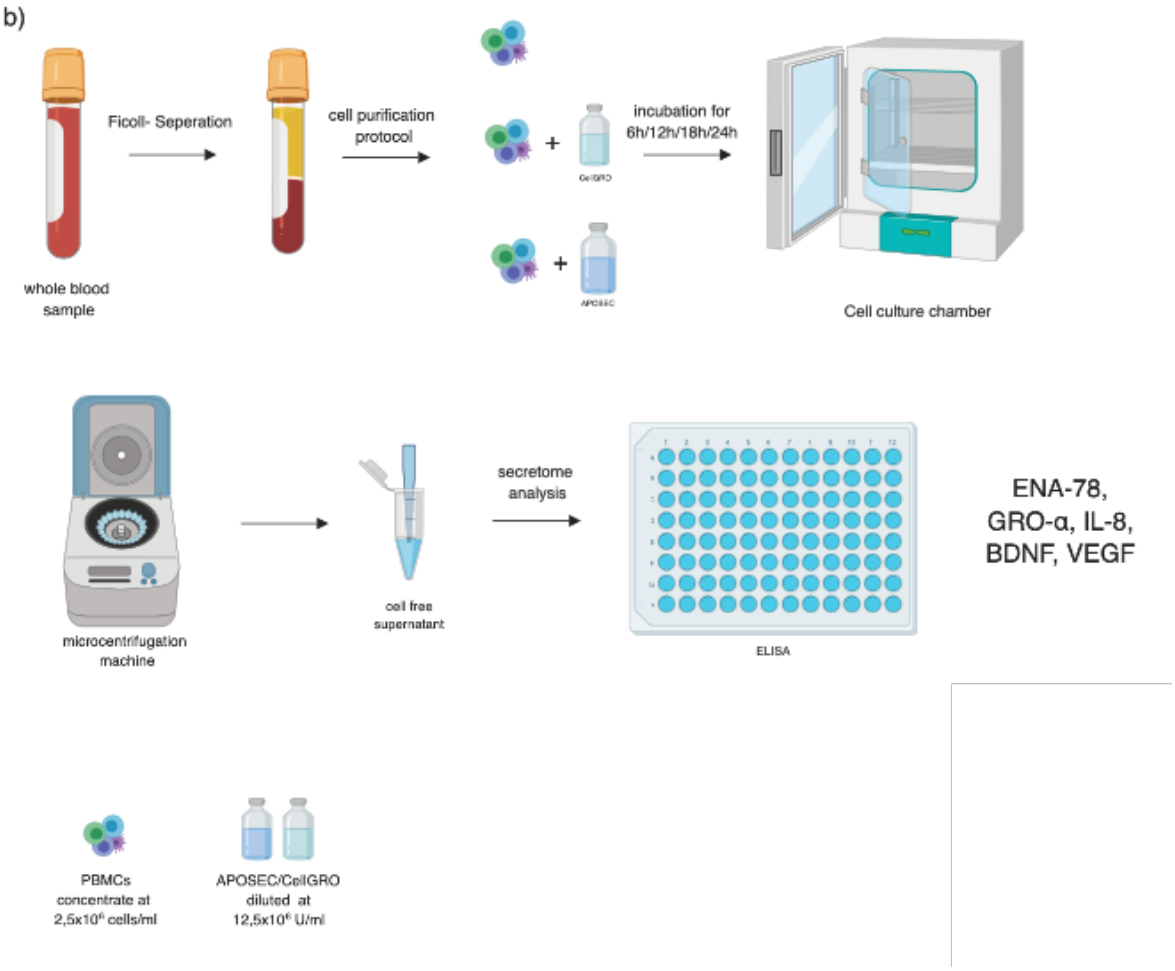

Figure 5.2 Assessment of changes in cytokine secretion over time. In this setting cells were obtained and handled as described in a) with the exception that cells received the tested compounds solely at a concentration of 12,5x106 cells/ml. Another difference was the removal of the cells from the cell incubator after preset time intervals (6h/12h/18h/24h). Evaluation of the cytokine levels was performing using ELISA. This figure was created with BioRender.com https://biorender.com last checked on 1st of May 2019

Furthermore, we wanted to investigate the influence of long – term hypoxia and hypoxic preconditioning on PBMCs and their secretion patterns, when exposed to GMP-APOSEC or CellGRO. Therefore, we employed two more settings. Cells were handled as described above. Firstly, after stimulation with either 12,5x10^6 cells/ml GMP-APOSEC or lyophilized CellGRO they were transferred to a hypoxia chamber – set at 1% oxygen in the atmosphere.

The incubation time in the hypoxia chamber was restricted to 1 hour. Afterwards they were transferred to regular cell culture and incubated for 23 hours adding up to a total of 24 hours exposure to the tested compounds.

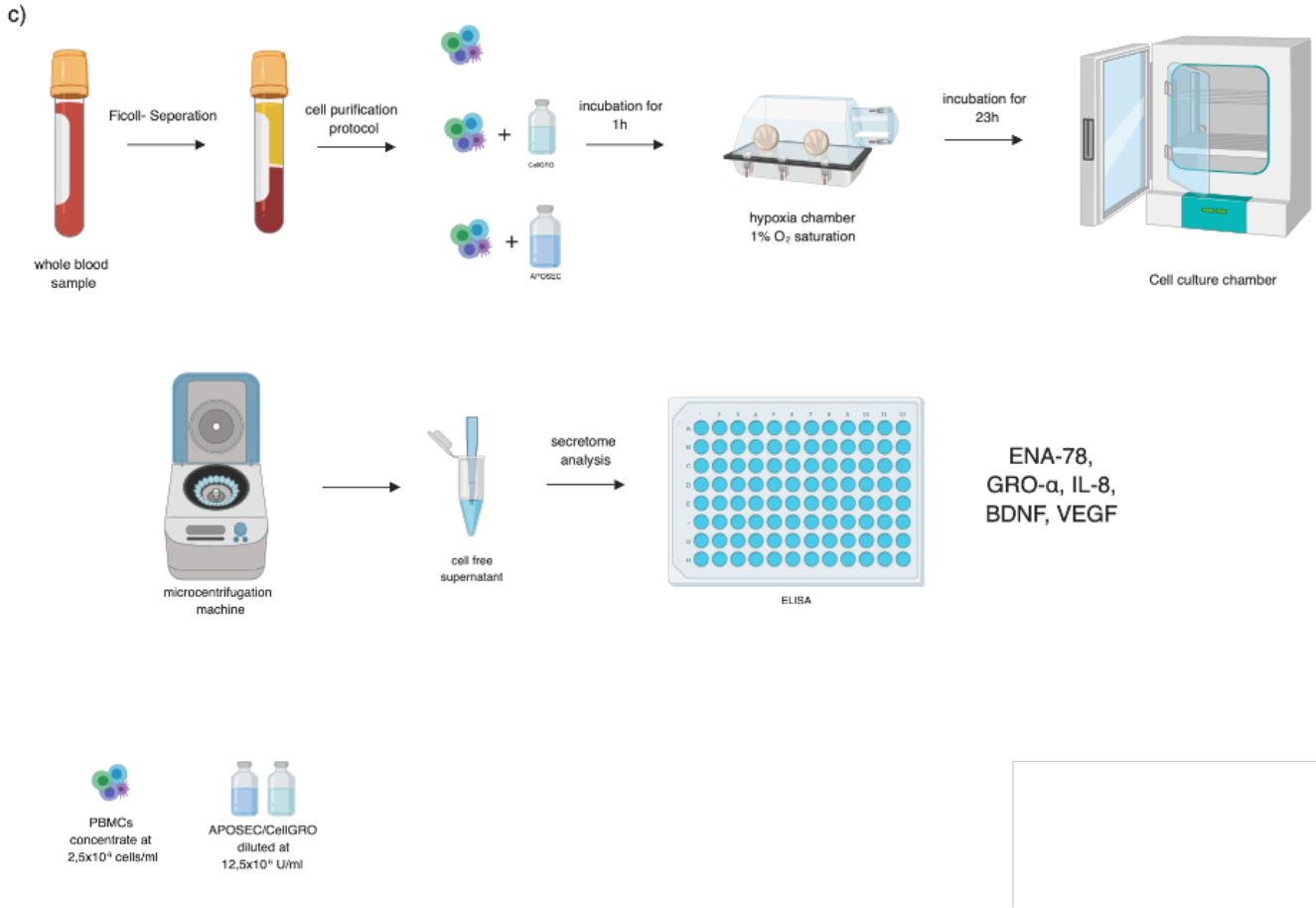

Figure 5.3. Hypoxic conditioning in cells pretreated with APOSEC/CellGRO prior to cell cultivation. After going through the same preparation steps as in b) cells were transferred to a hypoxic chamber – oxygen saturation was set at 1% in the atmosphere - for 1h/2h. Following that they were kept in cell culture until they had been exposed to the tested compounds for a total of 24h. Secretome analysis was performed as previously described. This figure was created with BioRender.com https://biorender.com last checked on 1st of May 2019

In the second setting, we deposited the cells in the hypoxic chamber – the same working conditions were applied as mentioned above - for 1 hour and added GMP-APOSEC and lyophilized CellGRO after removal from the hypoxic chamber, and before the transfer to regular cell culture settings. There they were kept for 23 hours again adding up to a total of 24 hours from isolation to further processing which involved separation of supernatants and conservation of cell pellets.

Cell number and concentration of treatment compounds did not differ in comparison to the methods described for the determination of time-dependency in cytokine secretion. The only changes in frame conditions were the implementation of the hypoxic chamber and the varying control times.

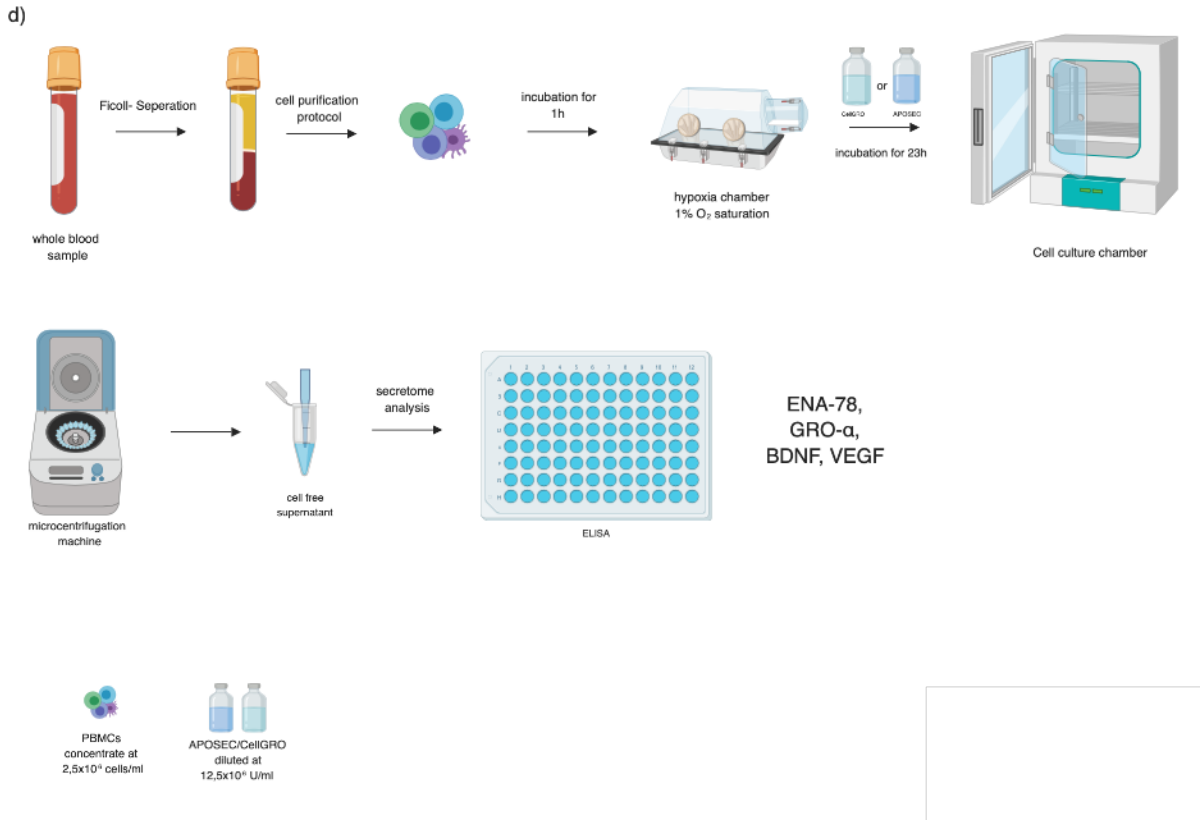

Figure 5.4 Application of APOSEC/CellGRO after PBMCs were exposed to hypoxic conditions. PBMCs were prepared as mentioned in b). Prior to addition of the tested compounds – at a concentration of $12{,}5x10^6$ cells/ml – samples were transferred to hypoxic chamber employing the same conditions as in c). After being exposed to the hypoxic environment cells received either APOSEC, CellGRO or were left untreated. The PBMCs were then transferred to a cell incubator for 23h. Secretome analysis for defined cytokines was performed as described above. This figure was created with BioRender.com. https://biorender.com last checked on 1st of May 2019

5.4 Enzyme – linked immunosorbent assay (ELISA)

Protein levels of IL-8, BDNF, VEGF, ENA-78, Gro-α, and IL1-RA - all Duoset kits, R&D systems, Minneapolis, USA- were quantified in the supernatants of PBMCs receiving either lyophilized GMP – APOSEC, lyophilized CellGRO or no treatment, following manufacturer's instructions.

In this study we used the cell culture supernatants as well as the ones that were preconditioned under hypoxic conditions for cytokine and growth factor detection. Analyzed protein concentrations complete our gene expression data and indicated whether changes in the gene expression actually lead to secretion of proteins.

First of all, the capture antibody specific to the antigen of interest was sealed on the plate. After dilution according to the manufacturers' protocol the capture antibody was incubated overnight at room temperature. This results in a coating of the Nunc-Immuno™ MicroWell™ 96 well plate with the capture antibody.
After 24 hours the plates were first washed and then blocked using blocking buffer – Reagent diluent which constitutes of 5g of Bovine Serum Albumin (BSA) diluted in 500ml of Dulbecco´s Phosphate Buffer Saline (DPBS) – for at least one hour to avoid unspecific binding.
After a further washing step, the samples and the predefined standards were applied on the 96 well plate. Standards and samples were tested in duplicates. During an incubation time of 2 hours the proteins in the sample and the standard bound to the specific capture antibody. Plates were then washed, and the biotin-linked detection antibody was added. Based on this step the term sandwich-ELISA was coined for this assay. It is important to mention, that the capture antibody binds to a different epitope than the detection antibody. Hence, the antigen-antibody complex of the capture antibody, that seals the antigen to the plate and the detection antibody, figuratively speaking, resemble a sandwich.
To visualize the amount of bound detection antibody induction of a fluorescent signal is needed. This was realized by the application of streptavidin-horse radish peroxidase (HRP). The HRP binds to the biotin which is linked to the detection antibody. To initialize the necessary color reaction colorless 3,3′,5,5′-Tetramethylbenzidine (TMB) was added. TMB facilitates hydrogen donation for the reduction of hydrogen peroxide (H_2O_2) to H_2O by streptavidin-HRP creating a blue color. This reaction is vulnerable to light exposition and was

therefore protected from direct light. The reaction was halted by the addition of 100 µl of 1 molar sulfuric acid. By photometric analysis with the Wallac Multilabel counter 1420 (PerkinElmer, Boston, Massachusetts, USA) the optical density was measured by a laser with a determined wavelength of 450nm. The deflection in combination with the defined standard curve defined the quantity of protein detected in the samples.

5.5 RNA-Purification

To isolate purified RNA, the pellets were first lysed with TRIzol Reagent, according to the lysate preparation protocol. The addition of chloroform to the sample, followed by centrifugation – at 14000 rpm for 20 minutes at 4°C - separated the solution into an aqueous phase containing RNA and a lower phenol-containing organic phase. .
The upper aqueous phase was transferred to a new tube, followed by addition of equal volume of 2- Propanol. After incubation for 24h at -20°C the tubes were centrifugated at the same settings as described above. Supernatants were discarded, and the RNA-pellet positioned at the bottom of the tube was washed with 1ml of 75% ethanol. After a further centrifugation step this washing process was repeated using 1ml 100% ethanol followed by a final centrifugation. After the supernatants had been discarded the RNA-pellets were air-dried for 5 minutes and re-suspended with 20µl H_2O. Purity and quality of isolated RNA was controlled by evaluation of 2µl of RNA stock with NanoDrop Microvolume Spectrometer. Obtained RNA was frozen away and will be suspect of studies to come.

5.6 Statistical analysis

All collected data was evaluated statistically using GraphPad Prism 5 (GraphPad Software Inc., California, USA). Data are expressed as mean +/- standard deviation (SD). Graphical description was displayed as box-plots - median, 1st and 3rd quartile are expressed, whiskers depict minimum and maximum values – and comparing column bar graphs.
As data did not meet requirements for parametric comparison, Mann-Whitney test (Two-way Anova) was applied for the comparison of concentration dependency. All other settings were statistically analyzed using One-way Anova. Dunn's Multiple Comparison Test or Bonferroni Test served as a post-hoc test to identify which specific means are significant from others. A two-sided corrected p-value < 0.05 was considered as statistically significant (s.), p- value < 0.01 as statistically highly significant (h.s.) and p-value $< 0{,}001$ as extremely significant (e.s.) respectively.

6. Results

6.1 Cytokine secretion varies with concentration of applied test compound

Application of lyophilized CellGRO and APOSEC to PBMCs resulted in differences of measured cytokine secretion when compared to PBMCs receiving none of the tested compounds. Our findings show that the highest levels of cytokine secretion were not measured when cells received the highest concentration of diluted compounds.
The PBMCs that had been treated with APOSEC and CellGRO diluted at different concentrations showed differences in measured cytokine levels. We analyzed whether significant changes in the paired groups (cell samples receiving APOSEC diluted at $25x10^6$ cells/ml vs cell samples receiving CellGRO diluted at $25x10^6$ cells/ml; samples receiving APOSEC diluted at $12,5x10^6$ cells/ml vs cell samples receiving CellGRO diluted at $12,5x10^6$ cells/ml; cell samples receiving APOSEC diluted at $2,5x10^6$ cells/ml vs cell samples receiving CellGRO diluted at $2,5x10^6$ cells/ml) occurred.

Levels of ENA-78 were highest in samples receiving APOSEC concentrated at $12,5x10^6$ cells/ml (Mean: 1830,2 pg/ml, SD: 95,15 pg/ml). In PBMCs treated with APOSEC diluted to $12,5x10^6$ cells/ml we measured higher ENA-78 levels when compared to PBMCs receiving CellGRO diluted at $12,5x10^6$ cells/ml (APOSEC group: 1830 pg/ml, SD: 95,153 pg/ml vs. CellGRO group: 1030,2 pg/ml, SD: 689,895 pg/ml, $p < 0,01$). Additionally we demonstrated significantly higher secretion values when comparing cells treated with APOSEC at $2,5x10^6$ cells/ml and PBMCs receiving CellGRO diluted at $2,5x10^6$ cells/ml (APOSEC group: 1136 pg/ml, SD: 437,3 pg/ml vs. control group: 312,6 pg/ml, SD: 536,6 pg/ml, $p < 0,001$). When comparing the other pairs, no significant difference was observed.

We made a comparable observation for the chemokine Gro-α. Highest concentrations of this cytokine were measured in the secretome of PBMCs incubated with APOSEC diluted at a concentration of $12,5x10^6$ cells/ml (Mean: 2237 pg/ml, SD: 702,9 pg/ml). When compared to the control group of cells that received CellGRO in a matching concentration the secretome showed significantly higher values (APOSEC group: 2237 pg/ml, SD: 702,9 pg/ml vs. CellGRO group: 409,8 pg/ml, SD: 519,1 pg/ml, $p < 0,001$). We did not see a significant difference when comparing the other two matched concentrations.

This did not apply for VEGF and IL-8. Highest levels of these cytokines were measured in PBMCs treated with APOSEC diluted at $2,5x10^6$ cells/ml.
For VEGF highest levels of cytokine concentration were measured in the cell incubated with APOSEC diluted at a concentration of $2,5x10^6$ cells/ml. In fact, values of VEGF were significantly higher when compared to PBMCs receiving CellGRO diluted to equivalent concentration (APOSEC group: 357,2 pg/ml, SD: 36,57 pg/ml vs. CellGRO group: 184 pg/ml, SD: 104,7 pg/ml, $p < 0,001$). For the cells receiving the tested compounds at one of the two other tested concentrations no statistically significant change in VEGF secretion could be observed in the tested supernatants.
For the chemoattractant cytokine IL-8 we detected significantly higher levels in the secretome of samples which received APOSEC at a concentration of $2,5x10^6$ cells/ml compared to matched CellGRO samples (APOSEC group: 1006 pg/ml, SD: 236,5 pg/ml vs. CellGRO group: 597 pg/ml, SD: 303,3 pg/ml, $p < 0,001$). Interestingly, secretion levels in PBMCs treated with APOSEC diluted at 12,5x106 cell/ml were not statistically significant from their paired CellGRO samples (APOSEC group: 945 pg/ml, SD: 280,8 pg/ml vs. CellGRO group: 984,2 pg/ml, SD: 188 pg/ml, $p > 0,05$ n.s.). However, for samples treated with APOSEC diluted at the highest concentration of $25x10^6$ cells/ml we observed significantly higher levels of IL-8 (APOSEC group: 631,2 pg/ml, SD: 87,1 pg/ml vs. CellGRO group: 100,6 pg/ml, SD: 12,2 pg/ml, $p < 0,01$).

IL-1RA levels were lower in the PBMCs receiving APOSEC compared to the CellGRO group. For cells treated with the compound diluted at a concentration of $12,5x10^6$ cells/ml we measured significantly lower levels in the samples receiving APOSEC (APOSEC group: 2713 pg/ml, SD: 682,1 pg/ml vs. CellGRO group: 5810 pg/ml, SD: 1009 pg/ml, $p < 0,01$), whereas PBMCs receiving either of the two other tested concentrations of $2,5x10^6$ cells/ml (APOSEC group: 1633 pg/ml, SD: 2486 pg/ml vs. CellGRO group: 3171 pg/ml, SD: 2611 pg/ml, $p > 0,05$ n.s) and $25x10^6$ cells/ml (APOSEC group: 213,4 pg/ml, SD: 55,53 pg/ml vs. CellGRO group: 189,4 pg/ml, SD: 100,5 pg/ml, $p > 0,05$ n.s) of tested compounds did not show statistically significant differences.

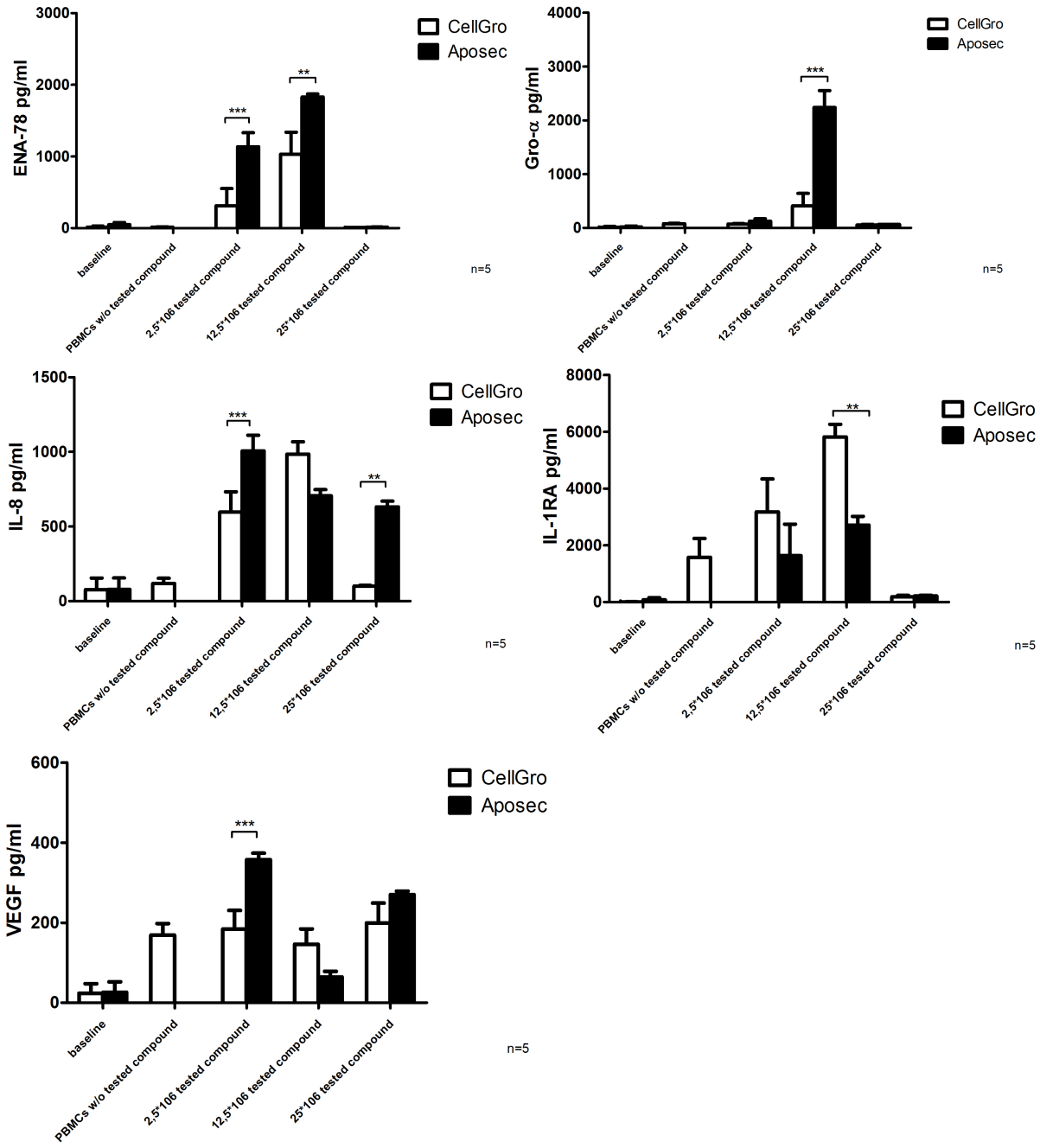

Figure 6.1 Concentration dependent cytokine secretion of PBMCs.

2,5x10^6 PBMCs were treated with either lyophilized APOSEC, lyophilized CellGRO or left untreated. The compounds were diluted to three different concentrations – 25x10^6 cells/ml, 12,5x10^6 cells/ml and 2,5x10^6 cells/ml – prior to 24-hour long incubation in a cell culture chamber. Furthermore, baseline values of the investigated cytokines were measured in the tested compounds. Supernatants were collected and analyzed for attenuated secretion of ENA-78, Gro-α, IL-1RA, IL-8 and VEGF.

6.2 Cytokine secretion occurs in a time-dependent manner

In order to assess the accumulation of secreted cytokines we set four timepoints at which cells were removed from cell culture after previously receiving either APOSEC or CellGRO – both set at a concentration of $12{,}5x10^6$ cells/ml – or none of the tested compounds.
After an interval of six hours one portion of the incubated cells were removed. In the text below the PBMCs receiving APOSEC will be referred to as APOSEC group, whereas the samples treated with either CellGRO or no treatment at all will be referred to as CellGRO group and control Group respectively.

Thus, we were able to demonstrate that PBMCs receiving APOSEC at the aforementioned concentration showed highest levels of cytokine secretion for ENA-78 after 12h of incubation. Levels of ENA-78 were significantly higher in the APOSEC group when compared to the CellGRO group and the control group. This observation was made for the samples removed after 12h (1753 pg/ml vs 580,6 pg/ml, $p < 0{,}001$ h.s.), 18h (1648 pg/ml vs 929,2 pg/ml, $p < 0{,}01$ s.), and 24h (1539 pg/ml vs 806,8 pg/ml, $p < 0{,}01$ s.) of incubation time.
Cytokine levels of ENA-78 were significantly higher in the samples treated with APOSEC compared to untreated samples that had been removed after 6h (627,2 pg/ml vs 13,75 pg/ml, $p< 0{,}05$ s.), 12h (1753 pg/ml vs 304 pg/ml, $p< 0{,}001$ h.s.) 18h and 24h. For the untreated samples we could not detect any levels of ENA-78 secretion at 18h and 24h. No statistical significance was measured when comparing the APOSEC group with the CellGRO group both removed at 6h of incubation time (627,2 pg/ml vs 102,0 pg/ml, $p > 0{,}05$ n.s.). Adding to that, we detected that cytokine levels of ENA-78 maintained high after initially peaking after 12h of cytokine secretion showing only little regression over the observed time.
Regarding Gro- α peak values were measured after 18h of cell culture. Highest secretion levels were detected in the samples co-incubated with APOSEC before transfer to cell culture. After 6h there was no significant distinction between the amount of this cytokine when comparing the APOSEC group to the CellGRO group (263 pg/ml vs 89,2 pg/ml, $p > 0{,}05$ n.s.) or the control group (263 pg/ml vs 131,6 pg/ml, $p > 0{,}05$ n.s.).
Significantly higher values for Gro-α were measured in the APOSEC groups when compared to the matching CellGRO groups incubated for 12h (668 pg/ml vs 116,2 pg/ml, $p<0{,}001$ h.s), 18h (1497 pg/ml vs 174,6 pg/ml, $p<0{,}001$ h.s.) and 24h (1195 pg/ml vs 145,8 pg/ml, $p < 0{,}001$ h.s.). Furthermore, PBMCs treated with APOSEC released significantly higher amounts

of Gro-α compared to untreated PBMCs samples incubated for 12h (668 pg/ml vs 140,2 pg/ml, p<0,001 h.s.), 18h (1497 pg/ml vs 139,2 pg/ml, p < 0,001 h.s.) and 24h (1195 pg/ml vs 141 pg/ml, p < 0,001h.s.).

In terms of IL-8 highest levels of cytokine secretion were measured in the APOSEC group after 6h. The amount of measured IL-8 secretion decreased over time in the samples treated with APOSEC and reached the lowest levels after 24h.

At all investigated timepoints we detected a difference in levels of IL-8 secretion in the APOSEC group when compared to the control group.

Values at 6h (2273 pg/ml vs 179,2 pg/ml, p < 0,001 h.s.), 12h (1777 pg/ml vs 294,6 pg/ml, p < 0,001 h.s.), 18h (1658 pg/ml vs 243,8 pg/ml, p < 0,001 h.s.) and 24h (1446 pg/ml vs 311,8 pg/ml, p < 0,001 h.s.) of incubation time were significantly higher in samples treated with APOSEC compared to untreated samples.

For PBMCs treated with APOSEC and removed after 6h of cell cultivation significantly higher levels were measured compared to the paired CellGRO group (2273 pg/ml vs 1644 pg/ml, p < 0,05 s.). No significant difference in the amount of secreted IL-8 between the APOSEC and the CellGRO groups was shown when analyzing the samples removed at 12h (1777 pg/ml vs 1998 pg/ml, p > 0,05 n.s.), 18h (1658 pg/ml vs 1924 pg/ml, p > 0,05 n.s.) and 24h (1446 pg/ml vs 1716 pg/ml, p > 0,05 n.s.) of cell culture.

Adding to the cytokines discussed above we also looked into the influence of the tested compounds on the release of growth factors such as VEGF and BDNF.

During the first six hours of incubation time significantly higher amounts of VEGF were detected in the APOSEC group compared to the CellGRO group (58,2 pg/ml vs 27,4 pg/ml, p < 0,05 s.) as well as the control group (58,2 pg/ml vs 27,8 pg/ml, p < 0,05 s.). Highest values of VEGF secretion were detected after 18h. At this timepoint there were no statistically significant differences in the amount of secreted growth factor between the groups (APOSEC group vs CellGRO group: 109,2 pg/ml vs 103,4 pg/ml, p > 0,05 n.s. and APOSEC group vs control: 109,2 pg/ml vs 120,8 pg/ml, p > 0,05 n.s). After 24h time in cell culture the control group showed significantly higher levels of VEGF compared to the APOSEC group (116 pg/ml vs 84,6 pg/ml, p < 0,01 h.s.). We did not measure significant differences in VEGF secretion between the APOSEC group and the CellGRO group after 24h cell cultivation (84,6 pg/ml vs 100,8 pg/ml, p > 0,05 n.s).

For BDNF we quantified higher levels in the samples treated with APOSEC compared to the other two groups. When set against each other the APOSEC group showed significantly

higher values compared to the CellGRO group after 6h (21 pg/ml vs 3,4 pg/ml, $p < 0,01$ h.s.), 18h (39,2 pg/ml vs 15 pg/ml, $p < 0,001$ e.s) and 24h (38,4 pg/ml vs 12,2 pg/ml, $p < 0,001$). This observation was also applicable when comparing the APOSEC treated group to the control group where we registered significantly higher level after 6h (21 pg/ml vs 0,8 pg/ml, $p < 0,001$ e.s.) 12h (13,6 pg/ml vs 0 pg/ml $p < 0,05$ s.) 18h (39,2 pg/ml vs 6,8 pg/ml, $p < 0,001$ e.s.) and 24h (38,4 pg/ml vs 4,4 pg/ml, $p < 0,001$ e.s.) timepoints. Peak values of BDNF secretion were reached in the APOSEC group after 18h.

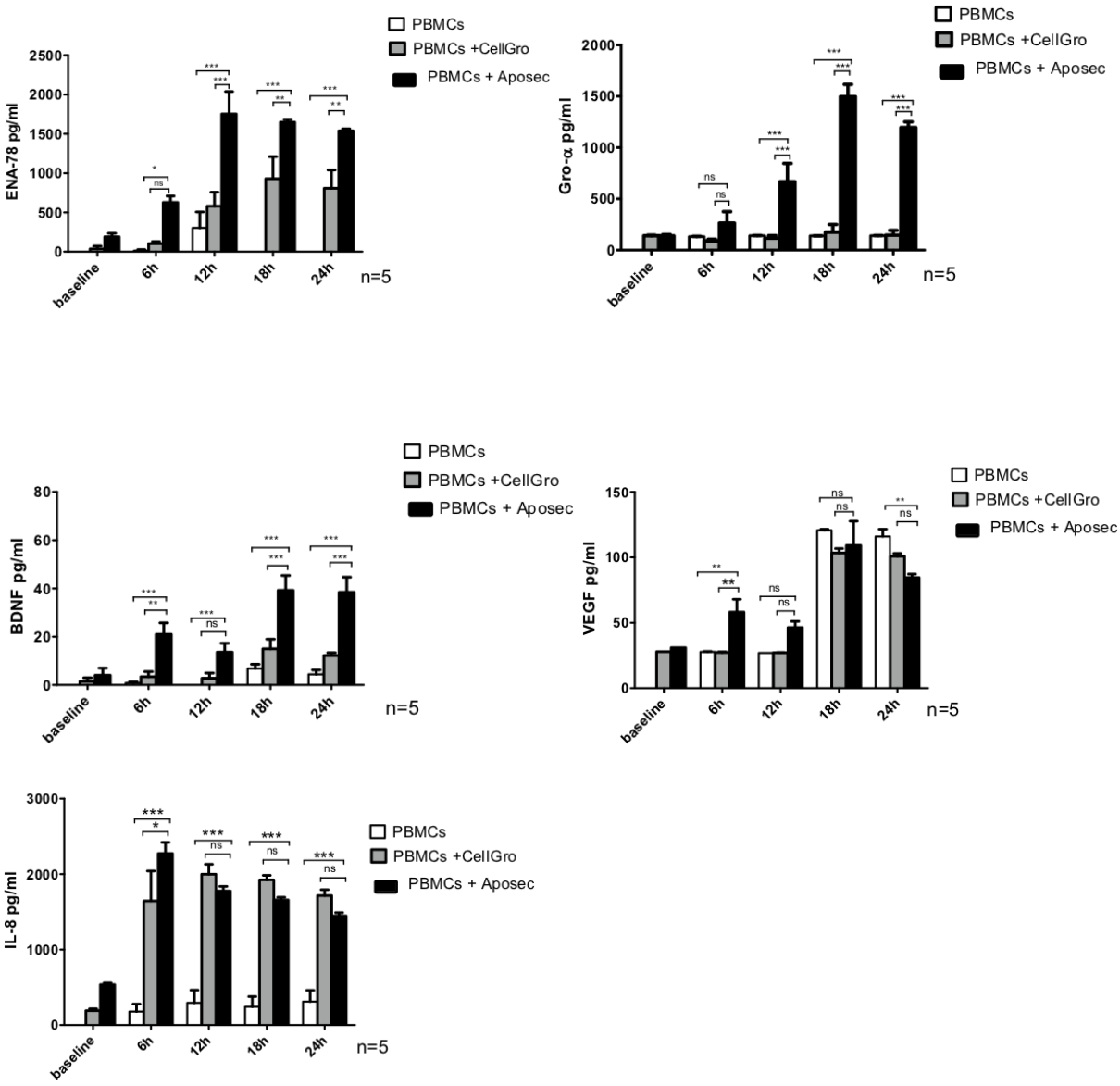

Figure 6.2. Time-dependent cytokine secretion of PBMCs incubated with APOSEC/CellGRO.

2,5x10^6 PBMCs were treated with either lyophilized APOSEC, lyophilized CellGRO or left in culture medium. The treatment compounds were diluted to a concentration of 12,5x10^6 cells/ml before incubation in a cell culture chamber. At defined six-hour intervals PBMCs were removed from cell culture and supernatants were obtained. Differences in secretion levels of ENA-78, Gro-α, IL-8, VEGF and BDNF are depicted. Furthermore, this column bar diagram shows at which timepoint highest values of respective cytokine were measured as well as what dynamic in terms of secretion pattern occurs in the first 24h after treatment with the tested compounds.

6.3 Cytokine secretion is higher in PBMCs incubated with APOSEC prior to exposure under hypoxic conditions compared to untreated control

For all the investigated cytokines secretion was lowest in the cells that did not receive any sort of treatment before a one-hour long incubation in a hypoxic chamber. Addition of APOSEC to cells before exposure to hypoxia for a defined period of time - 1h at 1% O2 saturation in the atmosphere - and subsequent incubation in cell culture under normal conditions for 23h resulted in the detection of significantly higher values of ENA-78 levels when compared to samples that did not receive any of the tested compounds (APOSEC group: Median = 2399 pg/ml, 1st quartile = 2153 pg/ml, 3rd quartile = 2467 pg/ml vs control group: Median = 56 pg/ml, 1st quartile = 51 pg/ml, 3rd quartile = 77 pg/ml, p -value: 0,0055 h.s.). Furthermore, measured cytokine levels for ENA-78 were higher in the APOSEC group compared to the CellGRO group (APOSEC group: Median = 2399 pg/ml, 1st quartile = 2153 pg/ml, 3rd quartile = 2467 pg/ml vs CellGRO group: Median = 768 pg/ml, 1st quartile = 339 pg/ml, 3rd quartile = 1168 pg/ml, p = n.s), albeit without statistical significance.
The secretome of PBMCs that had been pretreated with APOSEC showed significantly higher levels for Gro-α when compared to samples pre-treated with CellGRO (APOSEC group: Median = 1216 pg/ml, 1st quartile = 442,5 pg/ml, 3rd quartile = 1424 pg/ml vs CellGRO group: Median = 96 pg/ml, 1st quartile = 93 pg/ml, 3rd quartile = 10^6 pg/ml, p-value : 0,0058 h.s)No significant difference was detected when comparing the APOSEC treated cells to the ones receiving no treatment before exposire to hypoxia (APOSEC group: Median = 1216 pg/ml, 1st quartile = 442,5 pg/ml, 3rd quartile = 1424 pg/ml vs control group: Median = 126 pg/ml, 1st quartile = 122,5 pg/ml, 3rd quartile = 126 pg/ml, p = n.s.).

VEGF levels were highest in samples that received APOSEC. Untreated cells showed only low levels of cytokine secretion. For cells treated with APOSEC we measured significantly higher levels of VEGF in the cell-free secretome compared to untreated samples (APOSEC group: Median = 122 pg/ml, 1st quartile = 104 pg/ml, 3rd quartile = 227 pg/ml vs control group: Median = 24 pg/ml, 1st quartile = 20 pg/ml, 3rd quartile = 28 pg/ml, p-value : 0,0051 h.s). Comparison between the APOSEC group and the CellGRO group showed no statistical significance regarding secreted VEGF levels (APOSEC group: Median = 122 pg/ml, 1st quartile = 104 pg/ml, 3rd quartile = 227 pg/ml vs CellGRO group: Median = 43 pg/ml, 1st quartile = 35 pg/ml, 3rd quartile = 71,5 pg/ml, p = n.s.).

Cells challenged with the hypoxic hit without any treatment with the tested compounds showed the lowest levels of BDNF secretion. The addition of APOSEC before placement in the hypoxic chamber resulted in a higher release of BDNF (APOSEC group: Median = 108 pg/ml, 1st quartile = 93 pg/ml, 3rd quartile = 152 pg/ml vs control group: Median = 22 pg/ml, 1st quartile = 20 pg/ml, 3rd quartile = 37,5 pg/ml, p-value : 0,0066 h.s)). APOSEC treated samples had higher levels compared to samples that received CellGRO. However no statistical significance was detected between these two groups (APOSEC group: Median = 108 pg/ml, 1st quartile = 93 pg/ml, 3rd quartile = 152 pg/ml vs CellGRO group: Median = 48 pg/ml, 1st quartile = 43 pg/ml, 3rd quartile = 79 pg/ml, p = n.s.).

IL-8 secretion was highest in samples treated with CellGRO. Untreated PBMCs were very limited in their IL-8 secretion.
No statistically significant differences were measured when comparing the APOSEC group with the CellGRO group (APOSEC group: Median = 1928 pg/ml, 1st quartile = 1799 pg/ml, 3rd quartile = 1981 pg/ml vs CellGRO group: Median = 2147 pg/ml, 1st quartile = 1639 pg/ml, 3rd quartile = 2328 pg/ml, p-value = n.s.) and the APOSEC group with the untreated samples (APOSEC group: Median = 1928 pg/ml, 1st quartile = 1799 pg/ml, 3rd quartile = 2328 pg/ml vs control group: Median = 376 pg/ml, 1st quartile = 98,5 pg/ml, 3rd quartile = 538 pg/ml, p = n.s.).
To sum up, cells that had received APOSEC prior to hypoxic exposure showed higher cytokine secretion levels for ENA-78, Gro-α, VEGF, BDNF and IL-8 compared to untreated controls.

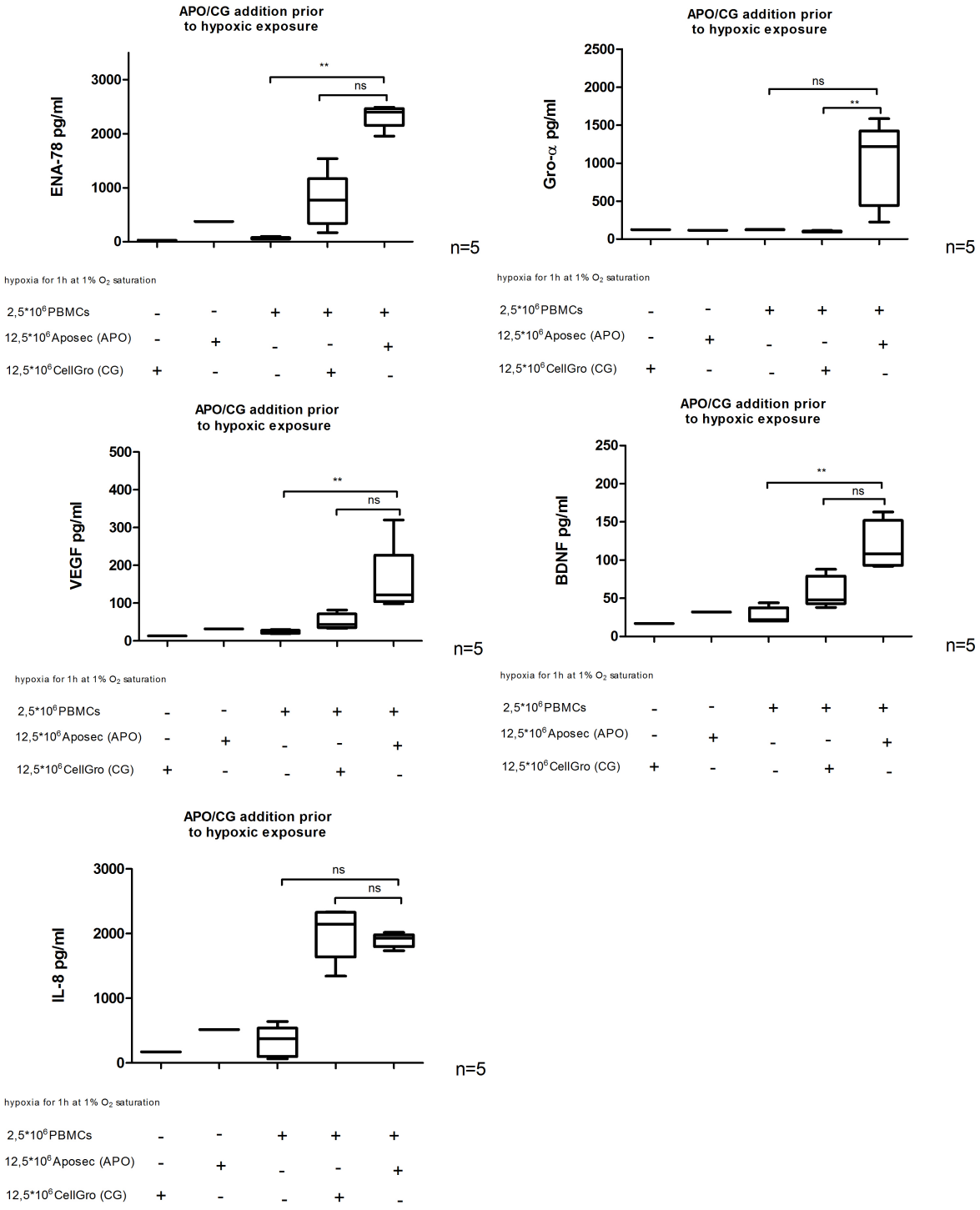

Figure 6.3 Exposure of PBMCs to hypoxia after receiving APOSEC/CellGRO.

2,5x10^6 PBMCs were treated with either lyophilized APOSEC, lyophilized CellGRO kept in culture medium. After treatment with lyophilized APOSEC or lyophilized CellGRO - both concentrated at 12,5x10^6 cells/ml – the cells were kept in a hypoxic cell culture chamber. After having been there for 1 hour cells were transferred to a normoxic cell culture until they hab been in contact with the tested compounds for 24 hours. Afterwards the cells were removed from the supernatants. Analysis of the supernatants for changes in secretion pattern of ENA-78, Gro-α, VEGF, IL-8 and BDNF was performed using ELISA.

6.4 Addition of APOSEC to PBMCs after hypoxic conditioing results in higher levels of cytokine secretion

Comparison of supernatants from PBMCs that were treated with APOSEC after the hypoxic incubation and untreated PBMCs after the hypoxic hit showed significantly higher levels of cytokine secretion for ENA-78 (APOSEC group: Median = 2525 pg/ml, 1st quartile = 2450pg/ml, 3rd quartile = 2580 pg/ml vs control group: Median = 349 pg/ml, 1st quartile = 11 pg/ml, 3rd quartile = 184 pg/ml, p-value: 0.0069 h.s.) and Gro - alpha (APOSEC group: Median = 2947 pg/ml, 1st quartile = 2671 pg/ml, 3rd quartile = 3687 pg/ml vs control group: Median = 76 pg/ml, 1st quartile = 74 pg/ml, 3rd quartile = 85,5 pg/ml, p-value: 0.009 h.s.). Although we also measured higher levels of VEGF and BDNF in the APOSEC receiving samples, neither BDNF (APOSEC group: Median = 67 pg/ml, 1st quartile = 38,5 pg/ml, 3rd quartile = 102 pg/ml vs control group: Median = 11 pg/ml, 1st quartile = 5 pg/ml, 3rd quartile = 28,5 pg/ml, p-value = n.s). nor VEGF (APOSEC group: Median = 86 pg/ml, 1st quartile = 43 pg/ml, 3rd quartile = 141,5 pg/ml vs control group: Median = 45 pg/ml, 1st quartile = 44,5 pg/ml, 3rd quartile = 46 pg/ml, p-value = n.s) showed a statistically significant difference in levels of cytokine secretion when compared to untreated control samples.

Adding to that, the secretome of PBMCs treated with APOSEC had higher values of all the cytokines we investigated when compared to PBMCs that received CellGRO prior to transfer into the hypoxic chamber. However, the values did not show statistical significance in terms of cytokine quantity for any of the investigated cytokines such as ENA-78 (APOSEC group: Median = 2525 pg/ml, 1st quartile = 2450pg/ml, 3rd quartile = 2580 pg/ml vs CellGRO group: Median = 1460 pg/ml, 1st quartile = 359,5 pg/ml, 3rd quartile = 1555 pg/ml, p = n.s), Gro-α (APOSEC group: Median = 2947 pg/ml, 1st quartile = 2671 pg/ml, 3rd quartile = 3687 pg/ml vs CellGRO group: Median = 359 pg/ml, 1st quartile = 108 pg/ml, 3rd quartile = 359 pg/ml, p-value = n.s.), VEGF (APOSEC group: Median = 86 pg/ml, 1st quartile = 43 pg/ml, 3rd quartile = 141,5 pg/ml vs CellGRO group: Median = 43 pg/ml, 1st quartile = 43 pg/ml, 3rd quartile = 44,5 pg/ml, p-value = n.s) and BDNF (APOSEC group: Median = 67 pg/ml, 1st quartile = 38,5 pg/ml, 3rd quartile = 102 pg/ml vs CellGRO group: Median = 37 pg/ml, 1st quartile = 11,5 pg/ml, 3rd quartile = 55,5 pg/ml, p-value = n.s.).

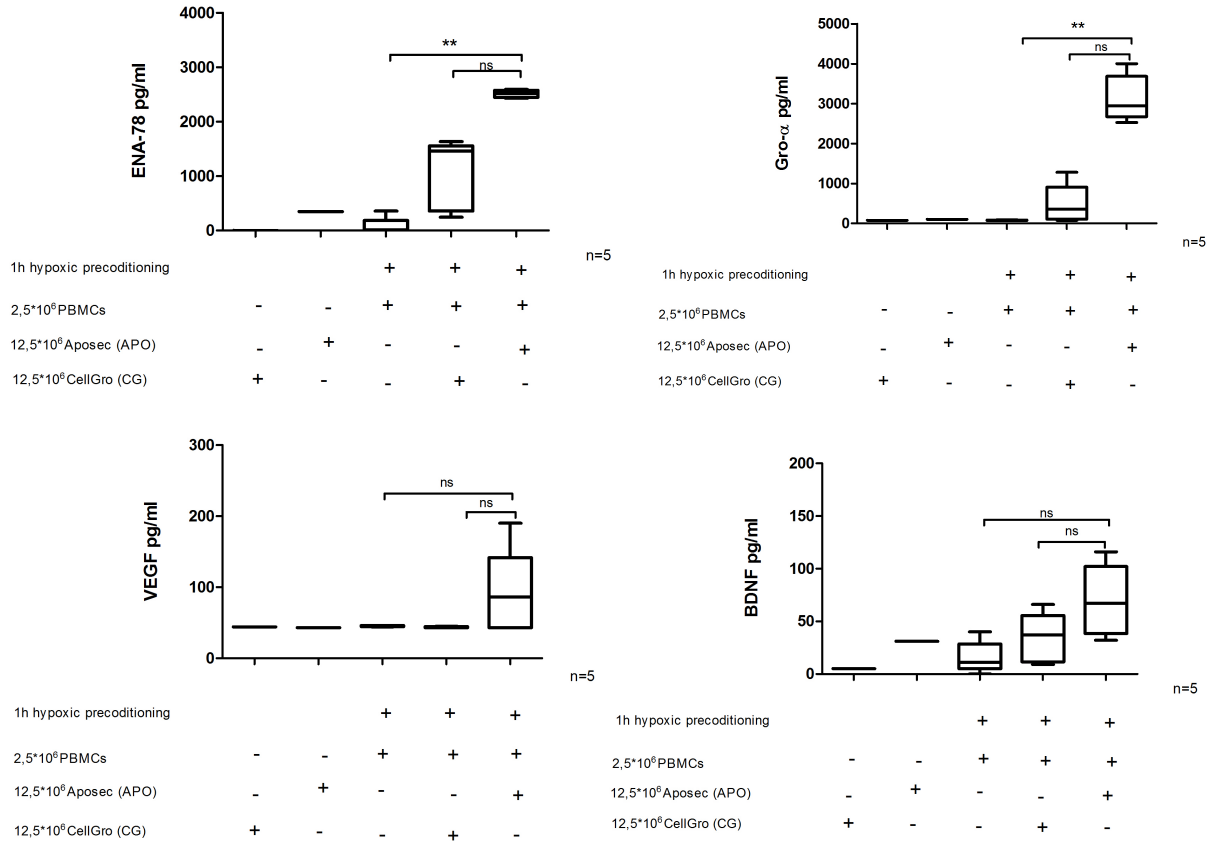

Figure 6.4. Influence of APOSEC on secretion pattern of hypoxic preconditioned PBMCs.

2,5x10^6 PBMCs were kept in a hypoxic cell chamber under hypoxic conditions – O2 saturation at 1% in the atmosphere - for 1 hour. Supernatants of cells treated with APOSEC had significantly higher amounts of ENA - 78, Gro-α and BDNF compared to untreated controls. For VEGF levels of measured cytokine secretion were not significantly altered.

7. Discussion

The implementation of cell-based approaches for therapeutic regenerative medicine has been proposed as a new staple for promoting tissue regeneration in damaged tissues. (1,12,103) After generating promising data from different in vitro cell culture and in vivo animal experiments employing stem-cells as the main driver for the regenerative properties (16,104), what proved to be a big obstacle was the translation of the generated knowledge into clinical application.(30)

It was the work of the group of Gnecchi et al. that demonstrated secreted paracrine factors derived from mesenchymal stem cells to be responsible for the tissue regenerative effects facilitated in an experimental model of acute myocardial infarction. (63,65) These results were to some extent the catalyst for numerous further studies leading to clarification of the biology of paracrine mechanisms and identification of the diverse underlying mechanisms of secretome-based therapy. (31,69,105,106) In spite of the many beneficial effects exerted by the stem cell secretome, there are considerable obstacles that have to be passed when employing the stem cell secretome. Stem cells represent a rare fraction of cells making their isolation and cultivation a difficult task and therefore neither simple nor easily practicable. PBMCs have been shown to release a variety of paracrine factors when entering programmed cell death. (82,94,107,108) The resulting cell – secretome exerts pleiotropic effects including inhibition of thrombus formation (109), promotion of angiogenesis (98), antimicrobial activity (99) and cytoprotection as demonstrated in a number of in vitro cell culture and in vivo animal experiments. (94) Compared to stem cells PBMCs are easily harvested and cultivated. Furthermore, the use of PBMCs allows for production of high quantities of cell-free secretome at very low financial costs as opposed to the stem cell secretome.

In this study we were able to show how addition of APOSEC to viable PBMCs influences their cytokine secretion pattern in vitro. The cytokines we investigated included ENA-78, Gro-α, IL-8, IL-1RA, VEGF and BDNF.

ENA-78 and Gro-α are both members of the chemokine family. Their functions include enhancement of chemotaxis for neutrophils with proangiogenic properties and wound healing. (110–113). IL-8 represents another chemokine with chemoattractant activity promoting neutrophil granulocyte migration and degranulation at inflammatory sites. (114,115)

IL-1RA is universally known as the antagonist binding to the proinflammatory cytokine IL-1. It is an anti-inflammatory cytokine important in host defense against endotoxin-induced injury. (116,117) VEGF and BDNF represent growth factors that take key roles in new vessel

formation (118,119) and maintenance of neuronal growth, differentiation, homeostasis and synaptic plasticity. (120–122)

Altogether these cytokines represent merely a fraction of the potent substances responsible for the many beneficial effect of the apoptotic secretome. They belong to the protein fraction present in the apoptotic secretome. The remaining constituents of the apoptotic secretome are made up of lipids, microparticles and exosomes. Wagner et. al demonstrated that in order to exert the beneficial effects facilitated by the cell free secretome all sub - fractions are necessary and therefore cannot be neglected. (92,98,123)

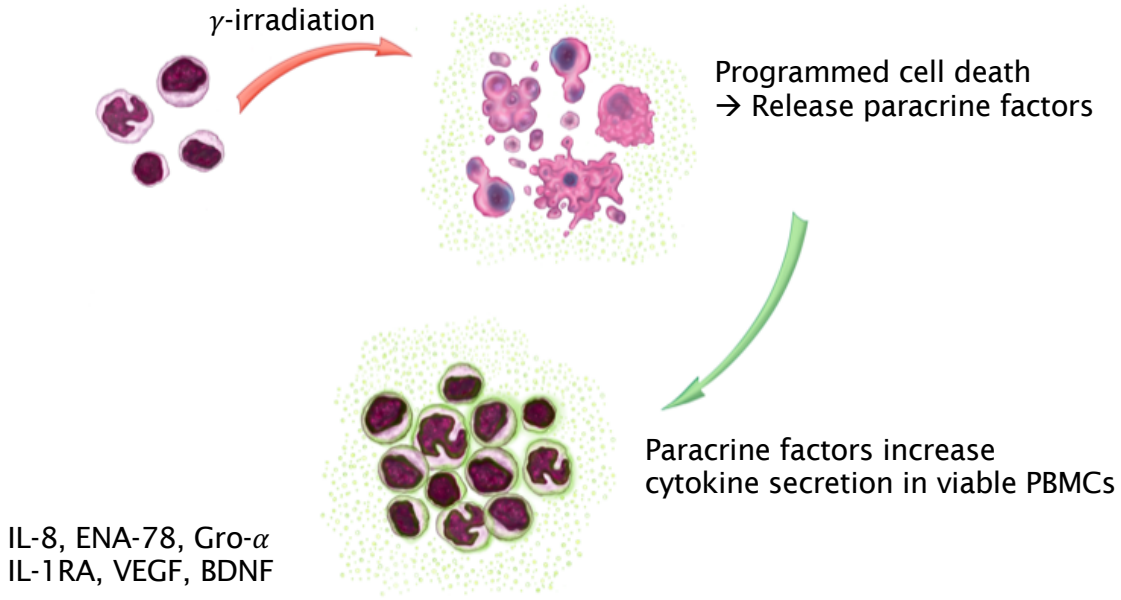

Figure 7.1. Paracrine factors released from irradiated PBMCs lead to cytokine secretion in viable PBMCs
Irradiation of PBMCs results in induction of programmed cell death. During the course of this process a plethora of biologically active substances - the cell secretome - is released. Amongst those are Growth factors, chemokines, interleukins and many more. In a paracrine manner, the secreted cytokines facilitate the release of further paracrine factors by PBMCs to further enhance the beneficial biological effects.
This figure was designed by Daniel Bormann using Sketchbook 7 Pro

In this pilot study we assessed the influence the apoptotic secretome of irradiated PBMCs has on viable PBMCs. More specifically we determined whether exposure to the apoptotic secretome resulted in an alteration in cytokine secretion.

When we exposed viable PBMCs with the apoptotic secretome of PBMCs we registered an increase of cytokine secretion. Out of the investigated cytokines, ENA-78 and Gro-α showed

highest alteration in secretion levels. Another interesting observation was that we registered only low levels of these two cytokines in the APOSEC samples used for stimulation of the viable PBMCs. This leads us to the conclusion that the addition of APOSEC to the viable PBMCs resulted in a significant increase of secreted ENA-78 and Gro-α from treated samples induced by the paracrine factors within the apoptiotic secretome. As already discussed, ENA-78 as well as Gro-α represent cytokines with chemotactic activity for neutrophil granulocytes which in return hold proangiogenic properties. Along with VEGF these factors may contribute to the enhancement of new vessel formation and augmentation of wound healing and tissue regeneration.

Adding to that we also registered low secretion of BDNF in the PBMCs treated with APOSEC.

Our data gives also an overview about the differences in secretion patterns of the investigated cytokines over time. For Gro-α, BDNF and VEGF we measured peak levels of cytokine secretion after 18h of incubation time with APOSEC whereas ENA-78 and IL-8 showed highest secretion levels at 12h and 6h respectively.

When exposing the cells to hypoxic conditions the PBMCs showed a decrease in secretory potency. However, the secretory capacity of PBMCs treated with APOSEC was not as diminished as compared to the untreated controls. Thus, the addition of APOSEC to PBMCs under hypoxic conditions served as a protective means and preserved their secretory capacities which in return can facilitate further cytoprotective actions in an autocrine as well as paracrine manner.

The aim of this project was to add to the present knowledge regarding APOSEC and the effects it exerts on viable PBMCs. In order to further understand the underlying mechanisms of the PBMCs and their interplay as origin of the potent apoptotic secretome as well as mediator of the beneficial effects more studies are necessary. Ultimately the goal is to transfer the collected information obtained by various members of our work group towards clinical implementation for which clinical trials in healthy and diseased patients are necessary.

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9. Abbreviations

AMI	acute myocardial infarction
Ang-1	Angiopoietin-1
APOSEC	Apoptotic Secretome
ASCs	adult stem cells
BDNF	Brain derived neurotrophic growth factor
BMCs	bone – marrow derived stem cells
BSA	bovine serum albumin
CD	cluster of differentiation
CFU-F	colony forming units with fibroblastic properties
CLPs	common lymphoid progenitors
CM	conditioned medium
CMPs	common myeloid progenitors
Cre/lox	cyclization recombination/locus of X
CSCs	cardiac stem cells
DPBS	Dulbecco´s Phosphate Buffer Saline (DPBS)
ELISA	enzyme linked immunosorbent assey
ENA-78	Epithelial-derived neutrophil-activating protein 78
EPCs	endothelial progenitor cells
EPO	erythropoietin
e.s.	extremely significant
ESCs	embryonic stem cells
FACS	Fluorescence activated cell sorting
G-CSF	granulocyte – colony stimulating factor
GMP-APOSEC	good manufacturing practice apoptotic secretome
Gro-α	growth-regulated oncogene -alpha
h	hours
H_2O_2	hydrogen peroxide
HBSS	Hank´s Balanced Salt Solution
HGF	Hepatocyte growth factor
HIF -1	hypoxia inducible factor 1
h.s.	highly significant
HSCs	hematopoietic stem cells

HRP	horseradish peroxidase
ICM	Inner cell mass
IL - 1-RA	Interleukin 1 – receptor antagonist
IL-8	Interleukin - 8
lacZ	lactose Operon Z
LAD	left anterior descendant
LNGFR	low affinity nerve growth factor receptor
MSCs	mesenchymal stem cells
µl	microliter
ml	milliliter
NK-cells	natural killer cells
n.s.	not significant
PBMCs	Peripheral mononuclear cells
PI3K	phosphatidylinositol 3-kinase
RNA	ribonucleotide-acid
s.	significant
Sca1	stem cell antigen
SCF	stem cell factor
SCID	severe common immunodeficiency disease
SDF-1	stromal cell derived factor 1
TGF-beta	transforming growth factor beta
TH1 cells	T-helper cell 1
TH2 cells	T -helper cell 2
TH17 cells	T-helper cell 17
TMB	3,3′,5,5′-Tetramethylbenzidine
TPO	thrombopoietin
VEGF	vascular endothelial growth factor
VEGFR2+	Vascular endothelial growth factor receptor 2+
Vs	versus
X-gal	5-Brom-4-chlor-3-indoxyl-β-D-galactopyranosid

10. Register of Illustrations and Figures

11. Curriculum Vitae

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