

THE SECRETOME OF MONONUCLEAR CELLS AS A THERAPEUTIC TOOL IN MYOCARDIAL INFARCTION AND MYOCARDITIS: AN EXPERIMENTAL STUDY

Doctoral thesis at the Medical University of Vienna
for obtaining the academic degree

Doctor of Philosophy

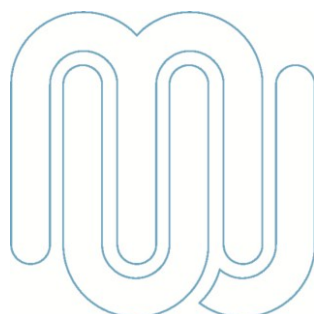
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“The art of healing comes from nature, not from the physician.
Therefore the physician must start from nature, with an open mind.”

Philippus Theophrastus Aureolus Bombast von Hohenheim, called Paracelsus
1493-1541

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ZUSAMMENFASSUNG

Die mikrovaskuläre Obstruktion (MVO) ist ein häufig beobachtetes Phänomen während des akuten Herzinfarktes und ist mit einer schlechten Prognose assoziiert. Aktivierte Thrombozyten und eine Engstellung der kleinen Blutgefäße sind Schlüsselfaktoren in der Entstehung einer MVO. APOSEC ist ein Präparat, das aus dem Kulturüberstand von peripheren mononukleären Zellen gewonnen wird und bereits in vorangegangenen Studien einen ischämischen Schaden im Myokard verringern konnte. Ziel dieser Studie ist es, den Einfluss von APOSEC auf die MVO im Herzinfarktmodell des Schweines zu untersuchen.

Der Zellkulturüberstand von bestrahlten, apoptotischen peripheren mononukleären Zellen (APOSEC) wurde Schweinen nach Induktion eines Herzinfarktes intravenös verabreicht. Die MVO wurde durch Magnetresonanztomographie-, Herzkatheter- und Elektrokardiogrammuntersuchungen bestimmt. Die Thrombozytenfunktion wurde *in vitro* und *in vivo* mittels ELISA, Durchflusszytometrie, Aggregometrie und Western Blot analysiert. Der Einfluss von APOSEC auf die Vasomotorik wurde in Nabelschnur-Endothel-Zellkulturen (HUVEC) und mittels Myographie-Experimenten mit isolierten Koronararterien-Ringen untersucht.

Die Verabreichung von APOSEC während eines Herzinfarktes führte zu einer signifikanten Reduktion der MVO. Thrombozytenaktivierungsmarker (P-selectin, CD40L, PF-4, TSP-1) waren in Plasmaproben von Tieren unter APOSEC Therapie niedriger als in Kontrolltieren. Die thrombozytenhemmende Wirkung von APOSEC wurde durch *in vitro* Experimente mit aufgereinigten humanen und porcinen Thrombozyten bestätigt. Als möglicher Mechanismus konnte eine vermehrte Phosphorylierung von VASP nachgewiesen werden. Zusätzlich waren die Plasmakonzentrationen vasodilatatorischer Mediatoren (NO, VIP, PGI₂) 40min nach APOSEC Verabreichung erhöht. HUVEC, die mit APOSEC kultiviert wurden, exprimierten verstärkt iNOS und phospho-eNOS. Unabhängig davon führte die Behandlung von isolierten Koronararterien-Ringen zu einer unmittelbaren, dosisabhängigen Vasodilatation in Myographie-Experimenten.

Die vorliegenden Versuche zeigen, dass APOSEC das Ausmaß der MVO während eines akuten Herzinfarktes verringert. Dies könnte eine Erklärung für die beobachtete, funktionelle Verbesserung in ischämischen Krankheitsbildern durch die Behandlung mit APOSEC erklären.

Die Myokarditis zählt zu den häufigsten Ursachen einer dilatativen Kardiomyopathie. Obwohl die Herzmuskelentzündung viele Parallelen zu einer „klassischen“ Autoimmunerkrankung besitzt, konnten Immunsuppressiva nur teilweise eine Verbesserung erzielen. In vorangegangenen Studien an Herzinfarktmodellern konnte eine therapeutische Wirkung einer Therapie mit parakrinen Faktoren peripherer mononukleärer Zellen gezeigt werden. Ein Mechanismus dieser Therapie basierte auf einer Modulation der Immunantwort während des Infarktes. Ziel der vorliegenden Studie war es, den Einfluß des Sekretoms von mononukleären Zellen (MNC Sekretom) auf die Herzmuskelentzündung in einem Myokarditismodell der Maus (EAM Modell) zu testen.

Zellkulturüberstand von mononukleären Zellen wurde Mäusen nach Induktion einer Autoimmunmyokarditis injiziert. Die entzündliche Antwort wurde mittels histopathologischer Aufarbeitung und ELISA Technik evaluiert. Der Einfluss des MNC Sekretoms auf hochaufgereinigte, humane CD4+ Zellen wurde *in vitro* anhand von Proliferations- und Apoptoseassays bestimmt.

Eine singuläre Applikation von MNC Sekretom führte zu einer deutlichen Reduktion des myokardialen Infiltrats in EAM Mäusen. Da es sich beim EAM Modell um eine CD4+ Zell abhängige Autoimmunerkrankung handelt, wurde der Effekt von MNC Sekretom auf CD4+ Zellen untersucht. Ko-Inkubation von T-Helfer-Zellen mit MNC Sekretom führte zu einer Caspase 8-abhängigen Induktion von programmiertem Zelltod.

Diese Daten zeigen, dass das MNC Sekretom die myokardiale Entzündung in einem Autoimmunmyokarditis-Modell reduzieren kann. Eine mögliche Erklärung für diese Beobachtung ist eine Induktion von Apoptose in CD4+ Zellen.

ABSTRACT

Microvascular obstruction (MVO) is a frequently observed phenomenon during acute myocardial infarction (AMI) and is associated with a poor prognosis. Platelets and vasoconstriction are considered key players in the pathophysiology of MVO. APOSEC, a compound produced from the secretome of apoptotic peripheral blood mononuclear cells, has previously been shown to attenuate the ischemic damage following AMI. The aim of this study was to evaluate the impact of APOSEC on MVO in a porcine closed chest ischemia/reperfusion AMI model.

Cell culture supernatants derived from irradiated, apoptotic peripheral blood mononuclear cells (APOSEC) were injected intravenously after induction of a myocardial infarction. Area of MVO was determined by magnetic resonance imaging, cardiac catheterization and by electrocardiography. Platelet function was monitored *in vitro* and *in vivo* by means of ELISA, flow cytometry, light transmission aggregometry and western blot. iNOS and phospho-eNOS induction in HUVEC cultures co-incubated with APOSEC was evaluated by western blots. Isometric circular wall tension of isolated coronary arteries was determined by myographic measurements.

Treatment of AMI with APOSEC resulted in a significantly improved microvascular perfusion. Platelet activation markers (P-selectin, CD40L, PF-4, TSP-1) were reduced in plasma samples, suggesting an anti-aggregatory capacity of APOSEC. This finding was confirmed by *in vitro* tests showing that activation and aggregation of purified platelets were significantly impaired by co-incubation with APOSEC. Vasodilatory mediators were systemically heightened 40min after administration of APOSEC in the porcine AMI model (NO, VIP, PGI₂). HUVEC co-incubated with the compound significantly upregulated iNOS and phospho-eNOS expression. Additionally, treatment of isolated coronary arterial segments with APOSEC resulted in a dilation of the vessel segments in a dose-dependent manner.

Our data give first evidence that APOSEC reduces the extent of MVO during AMI. This might explain the improved long-term outcome after APOSEC treatment in AMI.

Myocarditis is one of the major causes for dilated cardiomyopathy. Although the disease is considered to be associated with autoimmune phenomena, “classical” immunosuppressive regimes have only partially proven beneficial in clinical trials. We have recently evidenced that a high-dose application of paracrine factors obtained from mononuclear cells attenuates acute myocardial infarction. One mechanism responsible for the observed effect was a modulation of the detrimental inflammatory response following myocardial ischemia. The aim of this study was to determine the influence of MNC secretome on myocardial inflammation in a murine myocarditis model (EAM model).

Cell culture supernatants derived from murine mononuclear cells were collected and injected intraperitoneally after induction of myocarditis. The inflammatory response was determined by histopathological evaluation of explanted hearts and by ELISA technique. The impact of MNC secretome on CD4⁺ cells was evaluated by *in vitro* proliferation and cell viability assays.

Treatment of EAM mice with a single high-dose application of MNC secretome resulted in an attenuation of the myocardial infiltrate. Since the EAM model is a CD4⁺ cell dependent autoimmune disease we evaluated the effect of MNC secretome on purified human CD4⁺ cells. Co-incubation of MNC secretome with CD4⁺ cells led to a caspase-8 dependent induction of apoptosis.

In conclusion, our data show that MNC secretome reduces myocardial inflammation in an autoimmune myocarditis model. As a possible mode of action, an induction of apoptosis in CD4⁺ cells is suggested.

PART I – IMPACT OF APOSEC ON MICROVASCULAR OBSTRUCTION IN A PORCINE MODEL OF ACUTE MYOCARDIAL INFARCTION

Introduction

Stem cells and repair of the heart

For a long time the heart was thought to be an organ, which - once damaged - cannot be repaired. This view has been challenged at the beginning of this century. Autopsy studies on patients dying from congestive heart failure showed a 10–60 fold increase in mitotic figures in their hearts, although the absolute proportion was low (1). In addition, myocardial biopsies taken from sex-mismatched heart transplant recipients suggested an extracardiac origin of regenerative cells (2). These observations gave the basis for the idea to apply stem cells for regenerative purposes. After positive results in animal models the TOPCARE-AMI trial showed an improvement of left ventricular function after intracoronary application of bone-marrow-derived progenitor cells or blood-derived progenitor cells (3). These initial results were confirmed by others shortly thereafter (4, 5). Despite these encouraging data, stem cells have not found their way into routine clinical practice. The purification process is time-consuming and costly and a number of regulations have to be met. Another major reason for the failure of establishing stem cell therapy in the routine is a lack of involvement of the pharmaceutical industry. In contrast to research on e.g. small molecule drugs, it is a matter of debate if autologous stem cells have a value as intellectual property (6).

The Paracrine Paradigm

In the early days of stem cell research a differentiation of stem cells to cells with a local phenotype was thought to be the prevailing mode of action. By this way a regeneration of damaged tissue was assumed. However, only little evidence supports this theory. In fact, a complex interaction between stem cells and host tissue takes place. Although the molecular basis of this interaction is still a matter of research, the secretome of transplanted stem cells is considered the driving force behind this process. A key work for this change in paradigm was published in 2005 by Gnecci *et al.* They reported that medium previously “conditioned” by stem cells under hypoxic condition effectively reduced rates of hypoxia-driven cell death in cardiomyocytes. This finding was corroborated by *in vivo* experiments showing that the application of conditioned medium reduced infarct size and apoptotic index in a coronary artery ligation model (7). Shortly thereafter, other groups picked up this idea and showed that in fact most of the mechanisms seen in stem cell research can be explained by paracrine interactions (8, 9).

Lessons learned from secretome analyses

As the idea that the secretome of stem cells rather than the cells themselves mediates regeneration, detailed analyses of the secreted proteins came to the center of attention. Several groups tried to itemize soluble factors originating from stem cells by different techniques (10-12). The list of secreted cytokines, chemokines and growth factors is long and includes the following proteins - adapted from (13):

Angiogenic factor	M-CSF	IL-11	Transferrin
Follistatin (FST)	MCP-1	IL-12	receptor
BMP-4	MIP-3	IL-14	Osteoprotegerin 1
EGFR	IGFBP-1	IL-15	TIMP-1, TIMP-2
ENA78	IGFBP-2	IFN-g R	TGF-b3, MIF, TGF-
FGF-4	IGFBP-3	IP10	b2, LIF 1
FGF-7	IGFBP-4	OSM	
FGF-9	IL-1	PARC	
FGFR	IL-1b	PDGFR	
Flt-3 ligand	IL-3	PIGF	
GCP	IL-4	SCF	
G-CSF	IL-6	SDF-1	
GM-CSF	IL-7	TNF-a-I/IIR	
GRO	IL-7R	TGF-b-I/IIR	
LIF	IL-8	VEGF	

The secretome of stem cells is a rich source of pro-angiogenic, immunomodulatory and stem cell recruiting factors. A thorough evaluation of stem cell secretome in a substudy of the BOOST 2 trial revealed an interesting new perspective. Harvested bone-marrow derived stem cells and blood leukocytes from 15 patients were analyzed for their secreted factors (14). In a protein chip array study Wollert and colleagues showed that the secretome of both cell types was comparable for most of the evaluated proteins. Only 25 out of 174 factors were present in higher concentrations in bone marrow supernatants, and 10 factors were found in higher concentrations in peripheral blood leukocytes. In addition to that, both secretomes had similar effects on human coronary artery endothelial cell proliferation, migration and on cell sprouting in a mouse aortic ring assay. Our group was able to confirm these interesting observations by means of ELISA technique. Conditioned medium of viable as well as apoptotic peripheral mononuclear cells contained high amounts of proangiogenic factors e.g. IL-8, Gro-alpha, ENA-78, RANTES, HMGB1, PAI, MIF and VEGF (Table 1).

Table 1

soluble factors (ng/ml)	viable PBMC			apoptotic PBMC			sig.
	1.10 ⁶	2.5.10 ⁶	25.10 ⁶	1.10 ⁶	2.5.10 ⁶	25.10 ⁶	
IL-8	1.74 ±0.40	1.93 ±0.09	10.49 ±3.53	1.22 ±0.29	2.30 ±0.13	18.01 ±2.87	ns ns ¥
GRO-alpha	0.17 ±0.09	0.36 ±0.09	2.06 ±1.58	0.07 ±0.02	0.48 ±0.09	3.95 ±0.93	ns ns ns
ENA-78	3.41 ±1.34	29.93 ±3.41	34.89 ±16.33	3.93 ±1.43	37.86 ±12.73	108.86 ±27.88	ns ns ¥
MCP-1	1.66 ±0.65	0.47 ±0.21	0.27 ±0.00	0.76 ±0.19	0.74 ±0.17	0.27 ±0.00	ns ns ns
RANTES	8.32 ±0.18	18.62 ±3.21	37.63 ±2.72	4.01 ±0.05	22.25 ±3.64	51.58 ±4.44	ns ns ns
HMGB1	0.63 ±0.39	3.44 ±2.11	33.57 ±6.45	2.74 ±0.27	6.46 ±1.12	20.51 ±3.62	† ns ns
MMP9	4.14 ±0.91	14.59 ±2.75	29.46 ±8.29	0.99 ±0.16	3.61 ±0.59	19.35 ±5.34	† ‡ ns
sICAM-1	0.14 ±0.04	1.43 ±0.25	7.43 ±0.85	0.42 ±0.25	2.09 ±0.42	9.40 ±1.29	ns ns ¥
VEGF ₁₆₅	0.13 ±0.01	0.42 ±0.04	0.82 ±0.34	0.15 ±0.02	0.64 ±0.04	4.39 ±1.22	ns ns ¥
MIF	4.84 ±0.09	17.79 ±0.95	13.24 ±0.85	5.85 ±0.22	20.15 ±1.14	58.99 ±1.17	ns ns ¥
PAI-1	1.25 ±0.35	1.93 ±0.29	49.60 ±9.04	0.00 ±0.00	5.06 ±3.25	45.86 ±1.43	ns ns ns
IL-16	0.0 ±0.0	0.11 ±0.02	0.84 ±0.31	0.00 ±0.00	1.25 ±0.07	5.25 ±0.52	ns ‡ ¥
IL-1ra	0.35 ±0.09	0.52 ±0.17	2.16 ±0.96	0.13 ±0.04	0.41 ±0.17	6.43 ±1.33	ns ns ¥
IL-10	0.01 ±0.00	0.00 ±0.0	0.05 ±0.01	0.02 ±0.01	0.02 ±0.01	0.06 ±0.01	ns ns ns
IGF-I	0.00 ±0.00	0.01 ±0.0	0.03 ±0.02	0.00 ±0.00	0.01 ±0.01	0.03 ±0.03	ns ns ns
HGF	0.33 ±0.08	0.16 ±0.01	0.69 ±0.19	0.11 ±0.03	0.07 ±0.02	0.79 ±0.19	ns ns ns
FGF-2	0.56 ±0.02	0.53 ±0.00	0.59 ±0.01	0.48 ±0.01	0.53 ±0.02	0.55 ±0.02	ns ns ns
TGF-β	0.08 ±0.01	0.10 ±0.01	0.21 ±0.07	0.06 ±0.01	0.09 ±0.02	0.39 ±0.09	ns ns ns
SDF-1	0.17 ±0.0	0.19 ±0.0	0.22 ±0.03	0.16 ±0.01	0.15 ±0.07	0.12 ±0.04	ns ns ns
G-CSF	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00	ns ns ns
GM-CSF	0.00 ±0.00	0.00 ±0.00	0.07 ±0.02	0.00 ±0.00	0.00 ±0.00	0.08 ±0.02	ns ns ns

Table 1: Analysis of soluble factors secreted by non-irradiated cells and irradiated apoptotic PBMC (APOSEC).

Cells were incubated in three different cell concentrations for 24 hours. Supernatants were analyzed for cytokines, chemokines and growth factors (n=5). † p<0.05 1.10⁶ viable PBMC vs. 1.10⁶ apoptotic PBMC, ‡ p<0.05 2.5.10⁶ viable PBMC vs. 2.5.10⁶ apoptotic PBMC, ¥ p<0.05 25.10⁶ viable PBMC vs. 25.10⁶ apoptotic PBMC.

Table 1 shows content evaluation of the supernatant of apoptotic and viable PBMC - adapted from (15).

These results indicate that – although regenerative medicine has traditionally focused on stem cells – peripheral blood cells might mediate similar effects. Despite the fact that the therapeutic application of “conditioned” medium is nowadays an established concept, to date all attempts to identify a single effective agent in the secretome have failed. Blocking experiments have not led to sufficient results, as the elimination of single factors had no or only little impact on the effects mediated by the secretome (15, 16). Therefore, currently most groups propagate that the beneficial effects are mediated by a secretome in its entirety. This also implements that there might be more than one mode of action and that the regenerative potential of a secretome may be due to pleiotropic mechanisms.

Preceding experiments with apoptotic PBMC

Before the application of secretome derived from apoptotic peripheral blood mononuclear cells (PBMC) was considered, the impact of using apoptotic PBMC instead of stem cells was evaluated in preceding experiments (17). Mice systemically or intramyocardially treated with cultured, irradiated PBMC suspensions had significantly smaller areas of necrosis 72hrs after induction of acute myocardial infarction (AMI) when compared to controls. Furthermore, six weeks after AMI explanted hearts evidenced a reduced scar formation (25% vs. 6% of left ventricle; Figure 1) and improved left ventricular functional parameters in echocardiographic analysis.

Figure 1

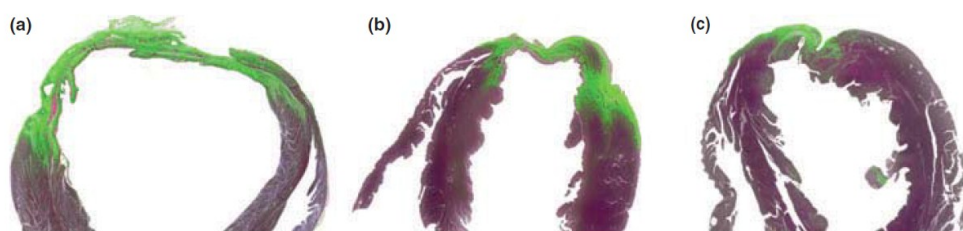


Figure 1 shows the extent of the myocardial damage following left anterior descending (LAD) ligation in a rat AMI model. Rats treated with apoptotic PBMC suspensions (c) had a significantly smaller area of scar formation when compared to untreated mice (a) and mice treated with viable PBMC suspensions (b); adapted from (17).

Histological evaluations revealed that the systemic application of cultured, apoptotic PBMC suspensions induced homing of regenerative cells to the myocardium. Apoptotic PBMC were shown to produce high amounts of IL-8 and MMP-9. Both chemokines play a key role in the recruitment of stem cells to damaged areas. Interestingly, incubation of fibroblasts with the secretome of apoptotic PBMC also led to a strong induction of IL-8 and MMP-9, giving first evidence that the secretome and not the cells might mediate the observed beneficial effects.

In a subsequent study by Lichtnerauer *et al.* it was shown that the intravenous and intramyocardial application of cultured, apoptotic PBMC suspensions led to changes of the extracellular matrix composition within the scar tissue. Animals receiving apoptotic PBMC suspensions evidenced an increment of elastic fibres in the transition zone between vital myocardium and fibrotic areas (18).

Role of APOSEC in ischemia/reperfusion

The idea to administer conditioned medium derived from apoptotic PBMC developed on the basis of the “Paracrine Paradigm” published in stem cell research. APOSEC is an acronym originating from APOptotic SEcretome. A standardized production process of APOSEC was established in our laboratory including the following steps: PBMC are isolated from healthy individuals and cultured for 24hrs in serum free medium. Cells are separated from the supernatant followed by steps of dialysis and lyophilization.

The first large animal study testing APOSEC in the setting of AMI was published in 2011. In a rodent coronary artery ligation model as well as in a porcine closed chest ischemia/reperfusion model, the intravenous infusion of APOSEC resulted in significantly reduced myocardial damage (12.6% vs. 6.9%, $p < 0.02$; Figure 2).

Figure 2

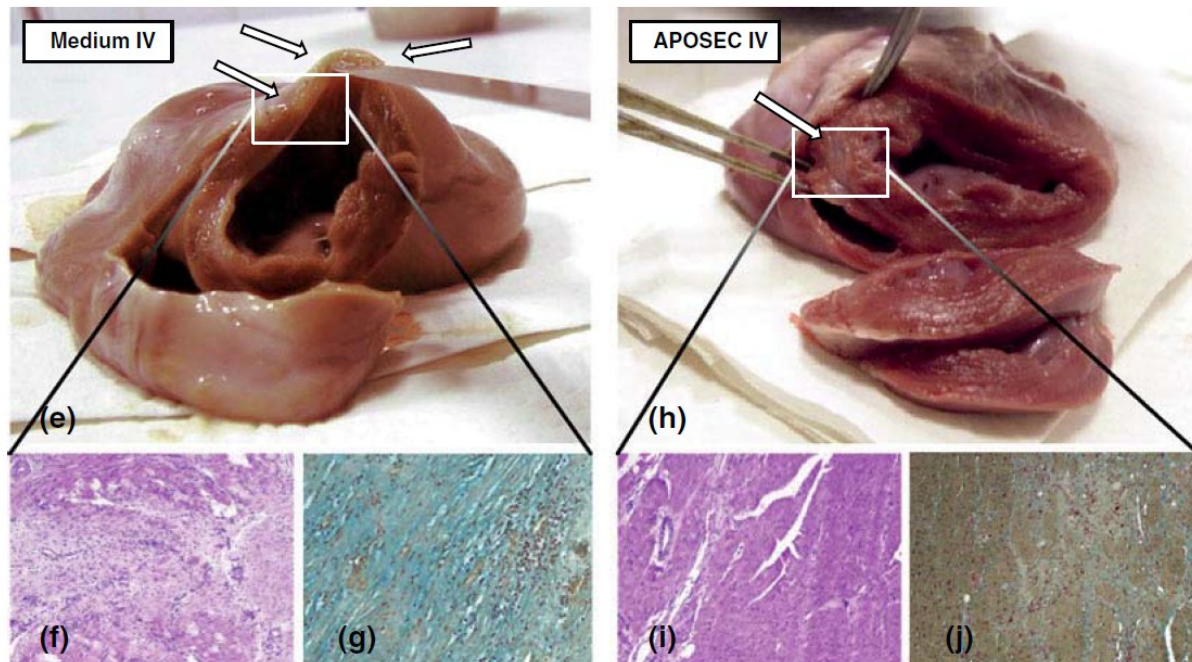


Figure 2 shows results of APOSEC treatment in a porcine AMI model. Pigs treated with APOSEC had smaller infarcts with less collagen deposition and more viable cardiomyocytes when compared to control medium pigs (H&E and Movat's pentachrome stainings); adapted from (15).

Additionally, magnetic resonance imaging (MRI) analysis of APOSEC treated pigs revealed improved haemodynamics (ejection fraction: 57.0% vs. 40.5%; cardiac output: 4.0 vs. 2.4 l/min) when compared to control animals. *In vitro* experiments indicated that co-incubation of cardiomyocytes with APOSEC led to an increment of pro-survival gene products (AKT, Erk1/2, CREB, c-Jun). Increased anti-apoptotic proteins (Bcl-2, BAG-1) could protect cardiomyocytes from starvation-induced cell death (15).

The no-reflow phenomenon – role of MVO in AMI

No-reflow is defined as a failure of reperfusion to an ischemic area after an obstruction has been removed or bypassed. In the setting of an AMI this means, that although coronary blood flow is re-established by an early intervention, a lack of perfusion on the microvascular level persists (microvascular obstruction – MVO). This phenomenon can be found in 22-50 percent of all AMI patients (19, 20) and the extent of MVO areas, has a significant impact on patients' prognosis (21, 22). Therefore, manipulating MVO is a main topic of research in cardiology. Pathophysiologically, MVO is a multi-dimensional phenomenon including platelets, the vessel wall and circulating leukocytes. Platelet activation has been shown to directly contribute to the impairment in coronary flow reserve and contractile function (23). Thus, inhibiting platelet function is thought to reduce no-reflow. This effect has been validated in clinical trials with GPIIb/IIIa inhibitors (24). Another impetus in treating no-reflow is a manipulation of the vasomotor state. Aspirates from occluded coronary arteries obtained during PCI were shown to contain factors of vasoconstriction and increased levels of apoptosis products (25-27). By antagonizing vasoconstriction, MVO can be effectively reduced.

This led to a recommendation of adenosine, verapamil or nitroprusside as a therapeutic option for no-reflow (28).

Aim of the study

Based on previously published findings of our group we sought to elucidate the impact of APOSEC on MVO during AMI. The restoration of blood flow on the microvascular level remains a major obstacle in cardiology as current pharmacological strategies of treating MVO are dissatisfactory. We employed a porcine closed chest reperfused infarction model to study effects of APOSEC on MVO. APOSEC was administered following the same protocol established in previous projects (17). The effect of APOSEC on the microvasculature was determined by MRI, electrocardiography (ECG) and heart catheterization analysis, following current recommendations (29). Since two major determinants of the no-reflow phenomenon are platelets and the vasomotor state we evaluated the impact of APOSEC on platelet function and vasodilation *in vitro*.

Material and Methods

Generation of APOSEC

APOSEC was produced according to a standardized protocol developed in our laboratory. PBMC were purified by Ficoll density-gradient centrifugation (GE Healthcare Bio-Sciences AB, Sweden). Cells were washed two times with HBSS and resuspended in CellGro serum-free medium (Cell Genix, Freiburg, Germany). Apoptosis was induced by irradiating cells with 60Gy. Cells were incubated for 24hrs at a concentration of $2.5 \cdot 10^6$ cells/mL in a humidified atmosphere (5% CO₂ and 37°C) and supernatants were collected. The conditioned medium was dialyzed against ammonium acetate (at a concentration of 50mM), sterile filtered, frozen and lyophilized. Aliquots of APOSEC were stored at -80°C until used for different experiments. APOSEC was pooled from four different donors for respective experiments. APOSEC was produced/applied in an allogeneic fashion: For animal experiments APOSEC was produced from donor pigs by direct heart puncture under sterile conditions. For *in vitro* experiments with human platelets and HUVEC PBMC were obtained from young healthy volunteers, patients suffering from insulin-dependent diabetes (APOSEC DM), or patients with congestive heart failure NYHA>III (APOSEC CHF) by venous blood withdrawal after obtaining informed consent (ethics committee vote: EK-Nr 2010/034; 2009/352). For *in vitro* experiments PBMC were cultured at a concentration of $1 \cdot 10^6$ cells/ml (platelet analysis) or $2.5 \cdot 10^6$ cells/ml (HUVEC cultures) in serum-free UltraCulture (Cambrex Corp., North Brunswick, NJ, US).

Comparative analysis of APOSEC derived from healthy donors, diabetes and CHF patients

Levels of IL-8, ENA-78 and VEGF (all Duoset kits, R&D Systems, Minneapolis, USA) in APOSEC preparations derived from healthy donors, diabetic patients and congestive heart failure (CHF) patients were determined following the manufacturer's instructions. Nitric oxide (NO) was measured by evaluating the decomposition product nitrite by a commercially available colorimetric assay kit (Abcam, Cambridge, UK).

APOSEC application in a porcine AMI model

All animal experiments were performed at the University of Kaposvar with a positive vote from the animal experiment committee (vote: 246/002/SOM2006, MAB-28-2005). Experiments were performed in accordance to the Guide for the Care and Use of Laboratory Animals by the National Institutes of Health (NIH Publication No. 85-23). Two different experimental settings were designed (Figure 3). Female "Large White" pigs weighing approximately 30kg received 75mg clopidogrel and 100mg acetylsalicylic acid one day before the intervention as a premedication. At the day of intervention animals were sedated (12mg/kg ketamine hydrochloride, 1.0mg/kg xylazine and 0.04mg/kg atropine) and put under general anaesthesia using isoflurane. The right femoral artery and the right femoral vein were punctured, heparin (200 IU/kg) was administered via the venous line and a 6Fr guiding catheter (Medtronic Inc., USA) was advanced via the arterial line to the left coronary ostium. A balloon catheter (diameter: 3.0 mm, length: 15 mm; Boston Scientific, Natick, USA) was inserted into the left anterior descending artery (LAD). The balloon was positioned after the second major diagonal branch and inflated to 4-6 standard atmospheres (atm). Forty minutes after the start of the LAD occlusion, APOSEC from $1 \cdot 10^9$ irradiated apoptotic porcine PBMC or lyophilized unconditioned serum-free cell culture (resuspended in 250 ml of 0.9% NaCl solution) was administered intravenously over 25min. After 90min occlusion, the balloon was removed again. Control coronary angiography (Ultravist contrast medium, Bayer Healthcare, Germany) was performed to prove the re-establishment of the epicardial blood flow and to exclude arterial injury caused by the procedure. Angiographic films were recorded and corrected TIMI frame counts and myocardial blush grades were evaluated later on in a blinded fashion. Corrected TIMI frame counts were determined according to the method of Gibson (30). Myocardial blush grading was evaluated as

previously published (31). Grades were defined as follows: grade 0 – failure of dye to enter the microvasculature; grade 1 – minimal myocardial contrast density; grade 2 – delayed entry and exit of dye; grade 3 – normal myocardial blushing. All pigs were ECG monitored throughout the whole experiment. An ECG rhythm strip was documented for every pig before LAD occlusion, after occlusion and after reperfusion. ST-segment resolution was calculated as an ST-segment decrease of >50% of the initial ST-segment elevation. Additionally, some pigs were monitored with a Holter ECG during ischemia and until 60min after reperfusion (Gepa-Med, Vienna, Austria). Euthanasia was performed by injection of saturated potassium chloride at 24hrs or 3 days after AMI induction.

MRI analysis

ECG-triggered MRI imaging was performed three days after AMI induction (Avanto, Siemens, Erlangen, Germany) using 0.05 mmol/kg gadolinium based contrast agent. MVO was determined by measuring areas of late hypo-enhancement within hyper-enhanced regions (i.e., "dark zones" within "bright" zones). Images used for MVO evaluation were recorded 10min after the administration of contrast agent (32). Infarcted areas were semi-automatically segmented by thresholding the left ventricular myocardium to the mean \pm 2*SD values of unaffected myocardium, independently by two blinded radiologists. Manual planimetry (QMass software, Medis, Leiden, the Netherlands) was used to define the area of MVO for each slice. Areas were multiplied by the slice thickness (8 mm) to get volumetric measurements. Results are given in volume values (cm³).

Human platelet isolation protocol

Blood draws were obtained from young healthy volunteers (ethics committee vote: EK-Nr 237/2004) by venipuncture using a wide-lumen needle (20 Gauge) to minimize mechanical induced stress. Whole blood was anticoagulated with 3.8% (w/v) trisodium citrate and platelet rich plasma (PRP) was obtained by centrifugation samples with 125g for 20min. PRP was further purified by means of sepharose column gel filtration to obtain highly purified human platelets. Experiments with porcine platelets were performed with PRP.

Platelet activation and aggregation

The impact of APOSEC on purified platelets was measured by flow cytometry and ELISA technique. Platelets were pre-incubated with APOSEC from 2×10^5 irradiated apoptotic PBMC for 10min. Platelets were stimulated for 5min with thrombin receptor-activating peptide (TRAP-6, BACHEM, Basel, Switzerland), adenosine diphosphate (ADP; Sigma-Aldrich, St Louis, MO, USA) or collagen (MoeLab, Langenfeld, Germany). Then platelets were stained with PE-labeled anti-CD62P, FITC-labeled anti-CD63 or FITC-labeled anti-CD40L antibodies (BD, NJ, USA) and evaluated on a FACSCalibur (BD, NJ, USA).

For aggregation experiments, platelets were stirred in the presence or absence of APOSEC from 2×10^5 cultured cells in an optical 4-channel aggregometer (490-4D, Chronolog Corp., Havertown, PA, USA) at 37°C for 5min to determine baseline absorbance. Then, TRAP-6, ADP or collagen was added and light absorbance was recorded over 10min. After 10min theophylline (300 μ M) and adenosine (500 μ M) were added to stop aggregation and supernatants were collected to measure amount of secreted soluble CD62P (Quantikine; R&D Systems, Minneapolis, MN, USA), soluble CD40L (Bender MedSystems, Vienna, Austria) and thrombospondin (TSP-1). ELISAs were performed following the manufacturer's instructions. TSP was determined by western blot as previously described (33). Samples were diluted in loading buffer (150mM Tris-HCl, pH = 6.8, 7.5% SDS, 37.5% glycerol, 0.1% bromophenol blue and 400mM of freshly added DTT) and heated to 95°C for 5min prior to loading. Proteins were separated on 8% reducing polyacrylamide gels and blotted to polyvinylidene difluoride membranes. Immunodetection was performed with anti-TSP-1 (NeoMarkers, Thermo Fisher Scientific, Fremont, CA, USA) at a concentration of 1:300. A peroxidase-conjugated secondary

antibody against mouse IgG and the SuperSignal West Femto (Pierce; Thermo Fisher Scientific, Rockford, IL, USA) was used as a detection system.

Quantification of intraplatelet VASP phosphorylation

Isolated human platelets were pre-incubated with APOSEC from 2×10^5 irradiated apoptotic PBMC together with different concentrations of prostaglandin E_1 (PGE_1) for 2min. Then ADP was added as an agonist for 5min. To determine the intracellular content of phosphorylated vasodilator-stimulated phosphoprotein (VASP), platelets were fixed in 1% formaldehyde for 10min and permeabilized with 0.5% Triton X-100. A monoclonal anti-phospho-VASP antibody, clone 22E11 (nanoTools, Teningen, Germany) was added for 45min. This antibody detects the phosphorylation site of VASP at serine 239. After a washing step, platelets were incubated with secondary FITC-conjugated anti-mouse IgG antibody (Becton Dickinson, Austria) for 30min and stained platelets were analyzed on a FACSCalibur (BD, NJ, USA).

Platelet activation markers and vasodilatory mediators during ischemia/reperfusion

Plasma samples (3.8% trisodium citrate tubes) were collected before LAD occlusion, 90min after occlusion, after reperfusion (240min) and 24hrs after AMI induction either via aspiration of the central line inserted in the femoral vein (0min, 90min) or by puncture of the superior vena cava (240min, 24hrs). Samples were evaluated for levels of sP-Selectin, TSP-1, PF-4, sCD40L, prostacyclin (PGI_2), vasoactive intestinal peptide (VIP) and nitric oxide (NO) by ELISA technique (Uscn, Wuhan, China; antibodies-online, Aachen, Germany; R&D systems, Minneapolis, USA). NO was determined by measuring the amount of oxidized metabolic product nitrite in the samples by a commercially available colorimetric assay kit (Abcam, Cambridge, UK).

HUVEC culture

3×10^5 primary human umbilical vein endothelial cells (HUVEC, CellSystems Biotechnologie, Troisdorf, Germany) were seeded in 6-well plates using endothelial cell growth medium (EGM-2, Lonza, Basel, Switzerland). Cells were incubated with APOSEC from 2.5×10^6 irradiated apoptotic PBMC or unconditioned control medium and cells were incubated for 60 minutes (phospho-eNOS detection) or 24 hours (iNOS detection) in a humidified atmosphere (5% CO_2 and $37^\circ C$). Then HUVEC were lysed in SDS-PAGE loading buffer, sonicated, centrifuged, and denatured before loading. SDS-PAGE was conducted on commercially available 8–18% gradient gels (GE Healthcare, Uppsala, Sweden). The proteins were then blotted onto nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). Immunodetection was performed either with a rabbit polyclonal anti-inducible nitric oxide synthase (iNOS) antibody (Cell Signaling Technology, Inc., Danvers, MA, USA), phospho e-NOS antibody (Cell Signaling Technology, Inc.), or a mouse monoclonal anti-GAPDH antibody (Acris, Herford, Germany). As a secondary antibody a horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse IgG antisera (both 1:10000; GE Healthcare) was used. Reaction products were detected by chemiluminescence with the ChemiGlow reagent (Biozyme Laboratories Limited, South Wales, UK) according to the manufacturer's instructions.

Myographic analysis of isolated coronary arterial rings

Hearts were obtained from untreated, domestic pigs purchased by a local butcher. They were brought to the laboratory in a modified Krebs-Henseleit buffer solution immediately after slaughtering. Coronary arteries were isolated from the heart and cut in 4mm thick rings. Each coronary segment was transferred to a temperature-controlled 10mL tissue bath containing a modified Krebs-Henseleit buffer solution. To measure circular wall tension, the rings were suspended between two L-shaped pins in a myograph. After approximately one hour, vessels were contracted

with endothelin-1 (30nM; Calbiochem, Darmstadt, Germany). Only segments responding to this treatment with a sufficient contraction were used for the following steps. APOSEC was added to the probes in different concentrations (dose escalation) and changes in arterial wall tension were measured. In some experiments iNOS inhibitor L-NG-Nitroarginine methyl ester (L-NAME) was added. For some experiments isolated coronary rings, co-incubated with APOSEC for 60min were processed following a standard protocol for immunohistochemical analysis. An antibody recognizing eNOS, phosphorylated at Ser1177 (Biorbyt, Cambridge, UK) was used. Histological slices were analysed on an Olympus AX70 microscope (Olympus Optical Co. Ltd., Tokyo, Japan) and captured digitally using Meta Morph v4.5 Software (Molecular Devices, Sunnyvale, USA).

Statistical analysis

All statistical analyses and graphic processing were performed using Graph Pad Prism software (Graph Pad Software, USA) or SPSS 18.0 (SPSS inc., United States). Results are depicted as means±standard error of the mean (SEM). Significances were calculated by student's t-test or repeated measures analysis of variance (ANOVA) followed by Bonferroni correction. In boxplot figures, whiskers indicate minimums and maximums, the upper edge of the box corresponds to the 75th percentile and the lower one the 25th percentile. A P-value less than 0.05 was considered as statistically significant (* indicates $p < 0.05$; ** indicates $p < 0.01$).

Results

In order to determine the impact of APOSEC on MVO two different experimental settings were designed (Figure 3). 16 pigs (APOSEC group n=9, control medium group n=7) were evaluated for MVO three days after AMI induction. In 13 pigs (APOSEC group n=6, control medium group n=7) ECG/Holter ECG, detailed heart catheterization examinations and repeated blood draws were performed.

Figure 3

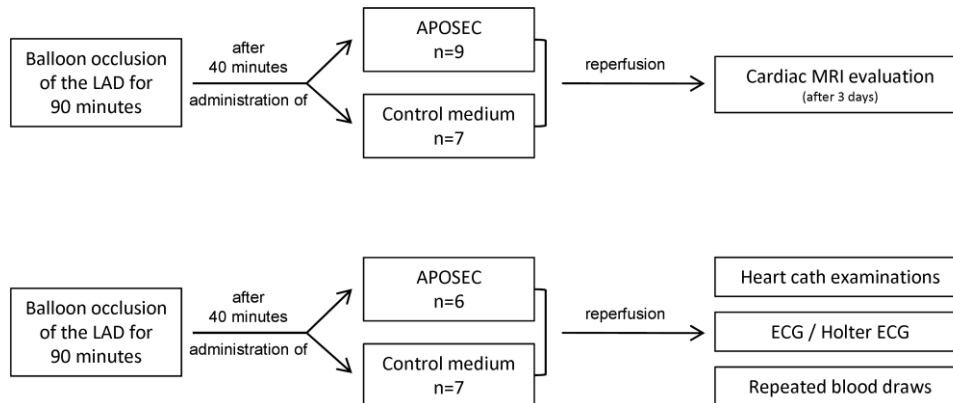
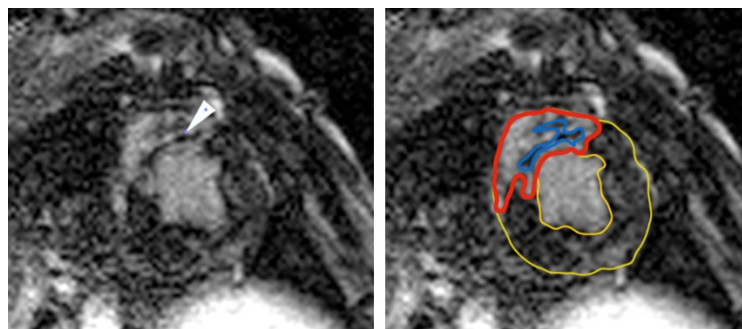


Figure 3 depicts the two experimental settings of the porcine AMI animal model.

APOSEC reduces MVO in a porcine AMI model

MRI analysis performed three days after induction of AMI revealed a significant impact of APOSEC on the microvascular patency. Areas of MVO were determined as areas of reduced contrast agent accumulation within hyper-enhanced areas (32). Qualitative and quantitative evaluations revealed an absent of or only small areas of MVO in APOSEC treated animals, whereas pigs treated with unconditioned medium had uniformly large areas of MVO (APOSEC: $0.3 \pm 0.1 \text{ cm}^3$; control: $0.8 \pm 0.1 \text{ cm}^3$; $p= 0.04$). One representative evaluation is depicted in Figure 4, detailed measurements are shown in Table 2.

Figure 4



Evaluation of MVO using late enhanced MRI analyses is shown in Figure 4. Hypoenhanced areas were calculated by planimetry.

Table 2

	group	MVO (cm ³)	Qualitative		group	MVO (cm ³)	Qualitative
#1	APOSEC	0	not visible	#10	control	0.86	small
#2	APOSEC	0.427	small	#11	control	0.76	small
#3	APOSEC	0	not visible	#12	control	1.04	small
#4	APOSEC	1.24	small	#13	control	0.26	very small
#5	APOSEC	0	not visible	#14	control	0.96	small
#6	APOSEC	0.56	small	#15	control	0.72	small
#7	APOSEC	0	not visible	#16	control	0.97	small
#8	APOSEC	0	not visible				
#9	APOSEC	0.91	small				

Table 2 shows MVO evaluations. APOSEC treated animals had significantly smaller areas of impaired microvascular perfusion when compared to control animals (n=7-9).

Parameters assessed during heart catheterization examinations confirm MRI analysis

The occurrence of MVO can also be visualized during heart catheterization examinations. For the corrected TIMI frame count the number of frames required for dye to reach a standardized distal landmark is determined. A correction factor is required to compensate for the different length of the three main coronary arteries (30). A low TIMI frame count indicates a sufficient blood flow in the small vessels, on the other hand a high TIMI frame count is associated with microvascular occlusion. The myocardial blush grade directly reflects tissue-level perfusion, as the accumulation of contrast agent in the intramyocardial capillaries are objectified by a grading system (34, 35). We could show that both parameters are impaired in control animals. Treating pigs with APOSEC significantly ameliorated corrected TIMI frame counts and myocardial blush grading (Table 3).

Table 3

	control	APOSEC	p-value
corr. TMI frame count	44.4±3.6	28.7±1.9	0.022
Myocardial blush grade	1.3±0.3	2.5±0.3	0.033

Table 3 displays angiographic evaluations. APOSEC treatment resulted in a significantly ameliorated corrected TIMI frame count and myocardial blush grading (n=6-7).

Bari scores and area at risk

To rule out possible bias in our study cohort we performed additional analyses. The Bypass Angioplasty Revascularization Investigation Myocardial Jeopardy Index (BARI score) is an angiographic-based score to evaluate the density and distribution of coronary vessels prior to an intervention (36). The Bari score was calculated based on LAD and circumflex coronary artery (CX) pre-occlusion angiograms and did not differ between our groups (APOSEC: 19.2± 1.0; control: 20.1±1.4; p=0.611; n=6-7; Figure 5).

The area at risk reflects the later extent of myocardial damage during the early phase of an infarction. Initially the area at risk comprises of still viable myocardium, which is gradually lost within the first hours to days if restoration of blood supply on the microvasculature level fails (37, 38). To confirm that ischemic areas were comparable in both groups we analyzed T2-weighted images of MRI analysis. In both groups areas of jeopardized myocardium were equal (APOSEC: 20.2±1.4; control: 22.9±2.2; p=0.294; Figure 5)

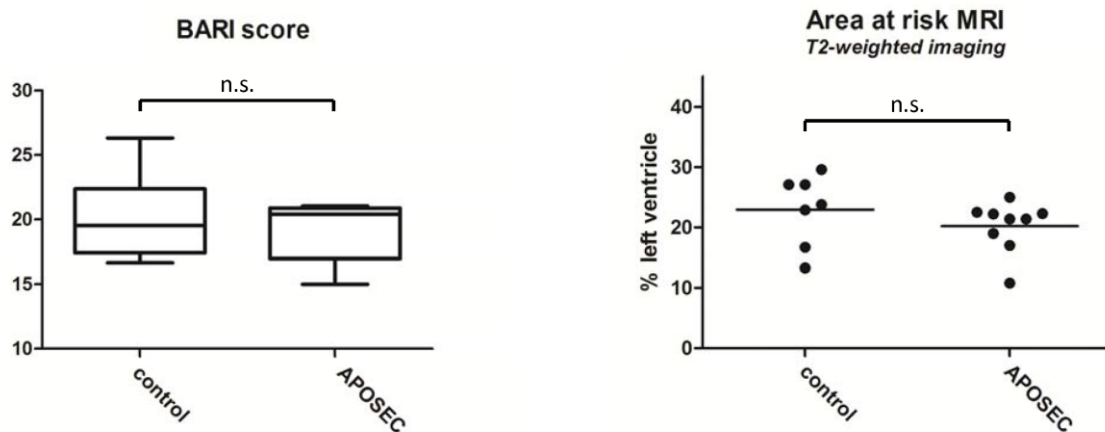
Figure 5


Figure 5 shows Bari sore and area at risk analysis (n=7-9). Cardiac catheterization films before the intervention showed no difference between groups indicating an even distribution of coronary collateralization (left). Area at risk determined on day 3 by MRI was comparable in both groups (right).

Haemodynamic monitoring

Left ventricular contractility was monitored in the pigs by heart catheterization after deflation of the occlusion balloon. $dP/dt/P$ (maximal rate of pressure rise dP/dt to the pressure P) is considered the gold standard in measuring left ventricular contractile capacity (39). In our cohort a tendency towards an improved pump function was observed after reperfusion, although this finding did not reach significance (APOSEC: $27.2 \pm 20.6 \text{ min}^{-1}$; control: $17.4 \pm 4.0 \text{ min}^{-1}$). Echocardiographic evaluations 24hrs after AMI induction confirmed this observation (Figure 6).

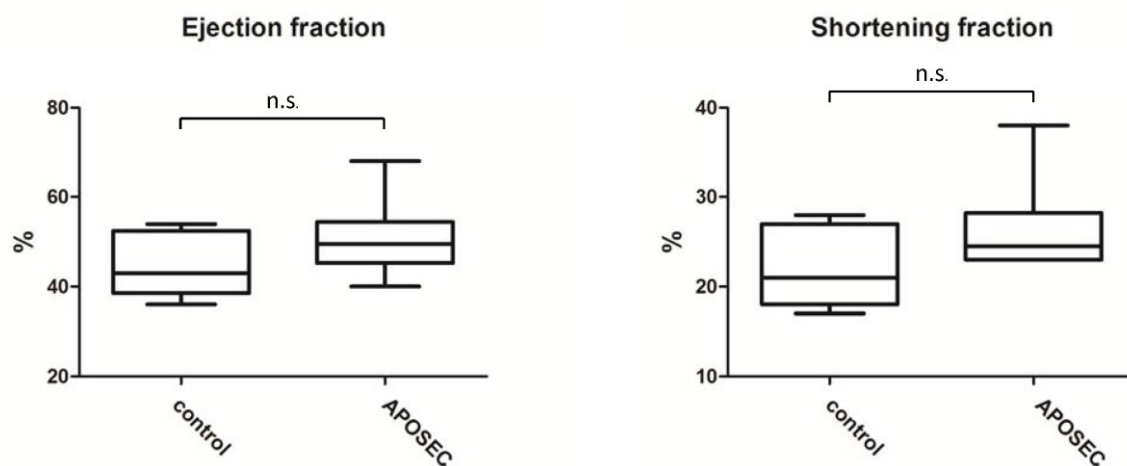
Figure 6


Figure 6 depicts functional evaluations 24hrs after APOSEC administration. Treated pigs showed a trend towards an improved ejection fraction and shortening fraction (Ejection fraction: APOSEC: 50.7 ± 3.8 ; control: 45.0 ± 3.3 ; $p=0.299$; Shortening fraction: APOSEC: 26.3 ± 2.4 ; control: 22.2 ± 2.1 ; $p=0.231$). However, these observations did not reach levels of significance (n=6-7).

Cardiac rhythm monitoring

ST-segment resolution is associated with a restoration of the blood flow in the microvasculature, since the supply of the electrical conduction system directly affects electrophysiological measurements. A higher amount of ST-segment resolutions was observed in the APOSEC group. In addition to that fewer episodes of spontaneously resolving ventricular arrhythmias (expressed in total number of extrasystoles, couplet, triplet, and ventricular tachycardias) were found during coronary occlusion and during the reperfusion period (Table 4).

Table 4

	ST-resolution	
	control	APOSEC
during occlusion	-	-
after reperfusion	1/7	4/6

	VES		couplet		triplet		VT	
	control	APOSEC	control	APOSEC	control	APOSEC	control	APOSEC
during occlusion	238.7 ±161.5	28.0 ±11.0	10.7 ±7.1	4.6 ±3.0	10.7 ±8.5	0.2 ±0.2	5.7 ±3.4	2.4 ±1.9
after reperfusion	92.3 ±31.0	49.0 ±35.8	18.8 ±8.6	8.0 ±5.6	4.8 ±3.3	3.4 ±2.0	3.2 ±1.9	3.4 ±2.4

Table 4 summarizes rhythmological analyses. ST-resolution was observed more frequently in the APOSEC group. Episodes of ventricular extrasystoles (VES), couplets, triplets and ventricular tachycardias (VT) were less frequent in APOSEC treated animals during occlusion and after reperfusion (n=6-7).

APOSEC inhibits platelet function *in vivo*

Platelets and their activation state are major contributing factors in the development of MVO following a myocardial infarction. Therefore, we evaluated secreted platelet function parameters in plasma samples obtained at different time-points. Systemic levels of sP-Selectin (sCD62P), TSP-1, PF-4 and sCD40L (sCD154) were lower in APOSEC treated animals when compared to pigs treated with unconditioned medium (Figure 7).

Figure 7

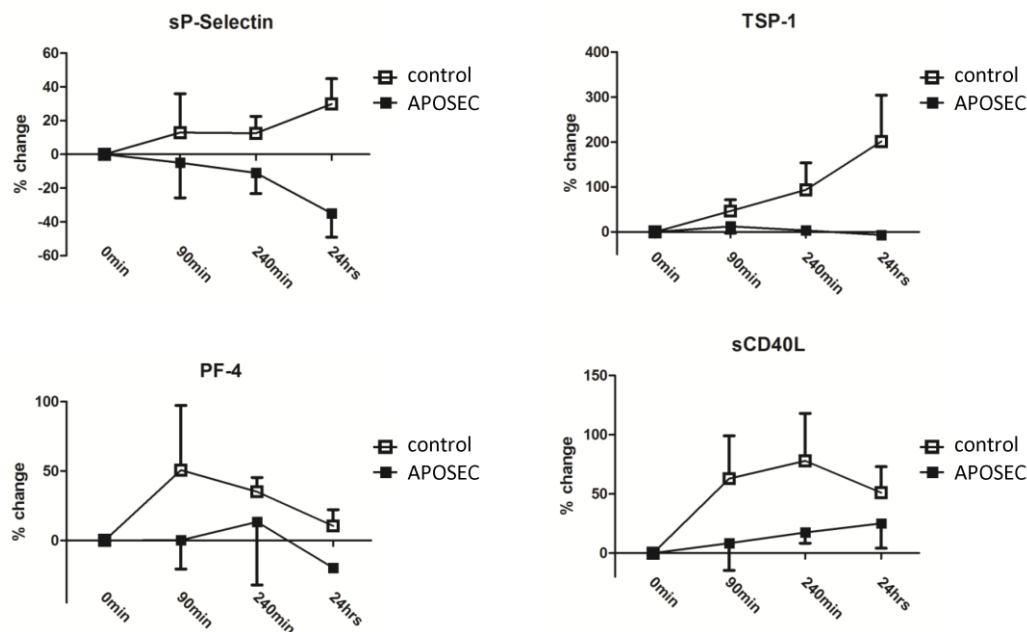
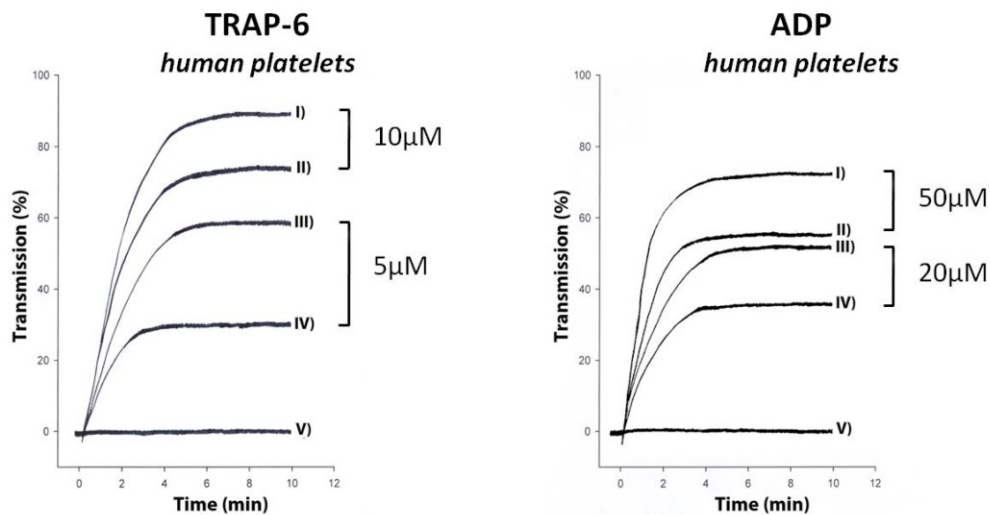


Figure 7 shows systemic levels of platelet aggregation markers in APOSEC treated pigs and control animals receiving lyophilized medium (n=6-7).

APOSEC inhibits platelet activation and aggregation *in vitro*

Next we evaluated the impact of APOSEC on platelet function *in vitro*. In a first experiment we tested whether APOSEC interferes with TRAP-6 and ADP-induced aggregation. Pre-incubation of highly purified platelets with APOSEC resulted in less clot formation as measured by light transmission aggregometry. This effect was observed when adding a maximal as well as a half-maximal concentration of the agonist (Figure 8).

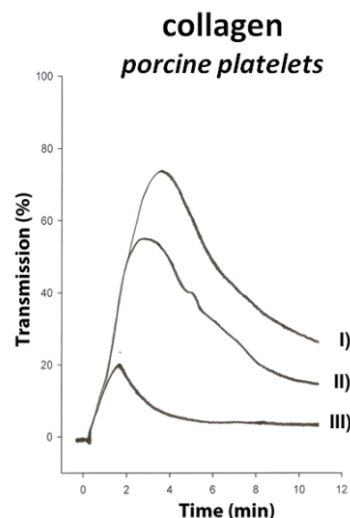
Figure 8



Aggregation experiments with human platelets are shown in Figure 8 (n=6). Clot formation of platelets activated with TRAP-6 and ADP was inhibited by adding APOSEC. This was shown for a maximal and a half-maximal activation dosage protocol. Left panel: I) TRAP-6 (10µM); II) TRAP-6 (10µM) + APOSEC 2*10⁵; III) TRAP-6 (5µM); IV) TRAP-6 (5µM) + APOSEC 2*10⁵; V) basal/APOSEC 2*10⁵; right panel: I) ADP (50µM); II) ADP (50µM) + APOSEC 2*10⁵; III) ADP (20µM); IV) ADP (20µM) + APOSEC 2*10⁵; V) basal/APOSEC 2*10⁵.

This experiment was repeated using APOSEC produced from pigs and porcine platelets (Figure 9). In both experimental settings – human and porcine – platelet aggregation was effectively reduced by the preparation, suggesting that the anti-coagulatory effect of APOSEC is a stable phenomenon throughout species.

Figure 9



Aggregation experiments using porcine platelets and porcine APOSEC showed similar results as the human experimental setting. APOSEC evidenced a dose-dependent inhibitory effect on clot formation. I) collagen (10µg/mL) II) collagen (10µg/mL) + APOSEC 1*10⁶ III) collagen (10µg/mL) + APOSEC 1*10⁷ (n=2).

In addition to the aggregation experiments we evaluated the impact of APOSEC on cell-surface bound and secreted activation markers. Levels of cell-surface bound CD62P, CD63 and CD40L were significantly decreased after treating platelets with APOSEC (Figure 10).

Figure 10

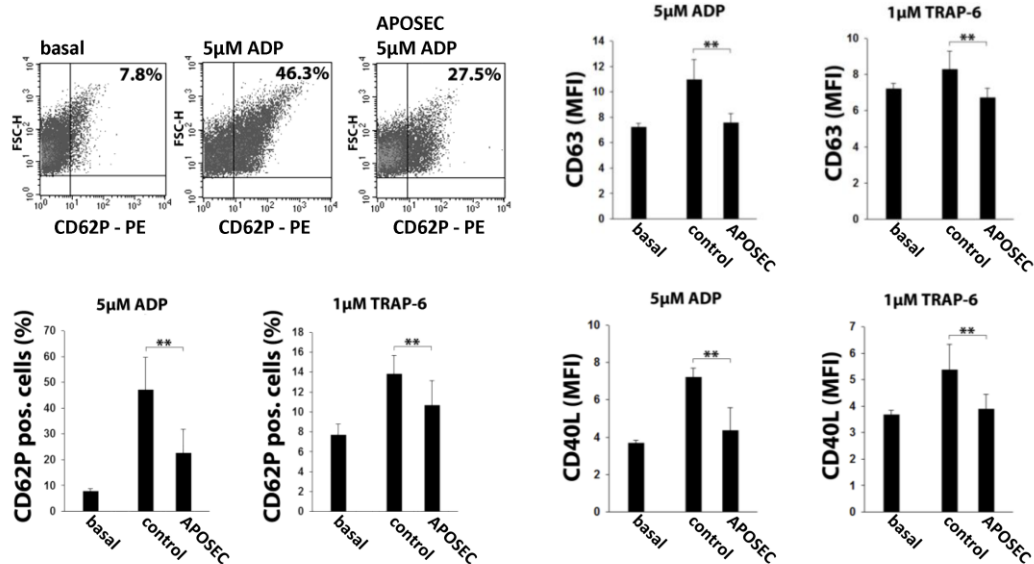
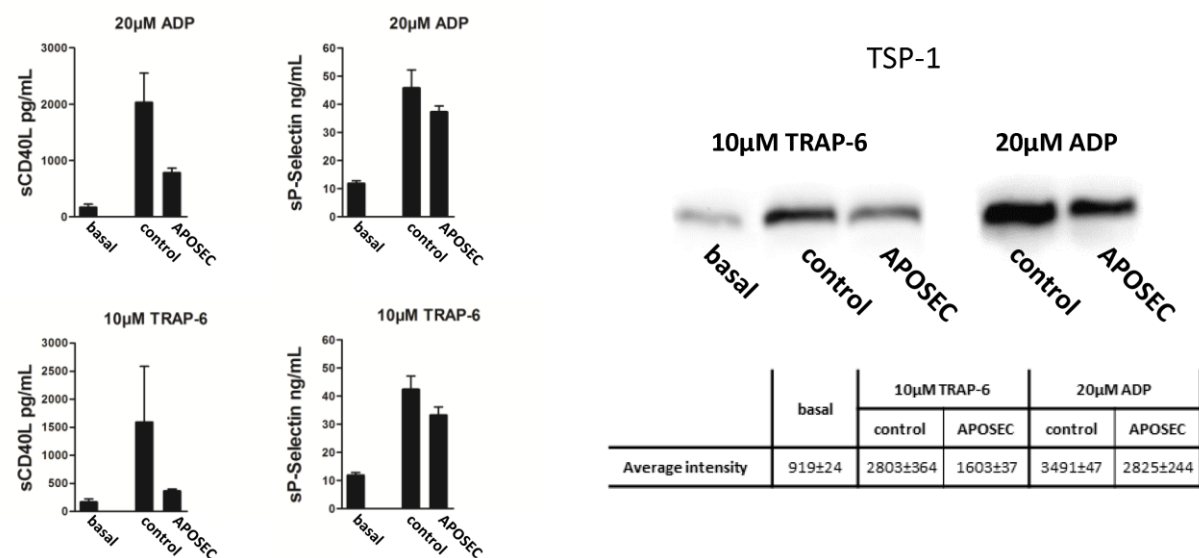


Figure 10 shows surface expression of platelet activation markers. APOSEC treatment of activated platelets resulted in reduced expression of CD63, CD62P and CD40L (n=8).

Levels of secreted activation markers (sCD40L, sCD62P and TSP-1) were lower after treating platelets with APOSEC as depicted in Figure 11. However, this finding did not reach significance due to the relatively small sample size.

Figure 11

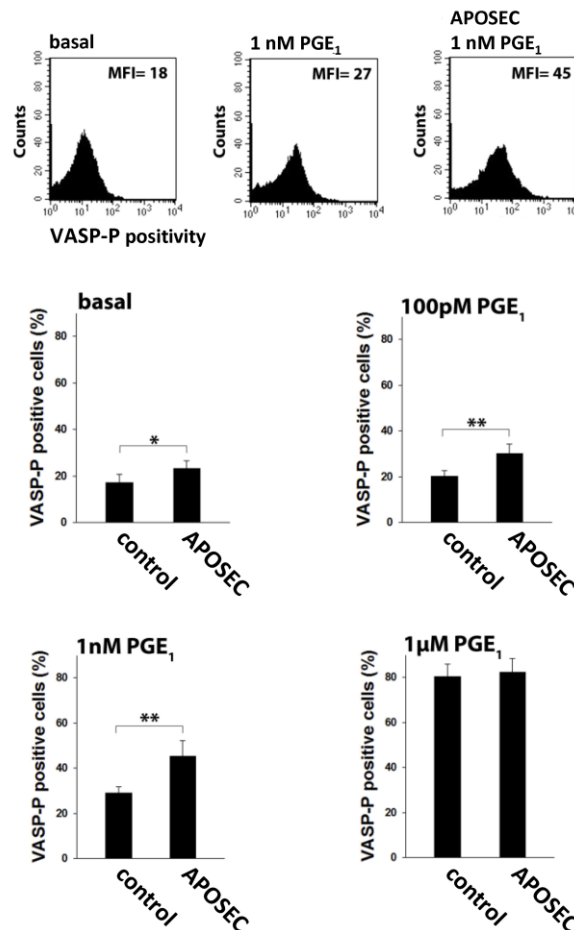


Evaluation of secreted platelet activation markers is shown in Figure 11. sCD40L, sP-Selectin and TSP-1 were lower in platelets treated with APOSEC when compared to control platelets (n=3).

Enhanced VASP phosphorylation by APOSEC

The phosphorylation of VASP is a major inhibitory pathway in platelets. We therefore evaluated if APOSEC had an influence on the phosphorylation state of VASP. Flow-cytometric analysis using an antibody that specifically detects the phosphorylation site at the Serin 239 revealed that incubation of platelets with APOSEC led to an increase of phospho-VASP (Figure 12). This effect was augmented by adding PGE₁, which is known to deploy its anti-coagulatory capacity via the VASP pathway (40).

Figure 12



The effect of APOSEC on VASP-phosphorylation is depicted in Figure 12. Platelets were analyzed for basal and PGE₁ induced VASP-phosphorylation in the presence and absence of APOSEC. Co-incubation with APOSEC (from $2 \cdot 10^5$ cells) led to significantly increased levels of phosphorylated VASP (n=8).

APOSEC induces coronary vasodilation

Since APOSEC consists of a myriad of proteins, it is most likely that more than one mode of action is responsible for the observed reduction of MVO during AMI. Besides platelets, vasomotor effects are thought to play a major role in the development of microvascular dysfunction during AMI. We therefore investigated, if APOSEC had an impact on the vasomotor tone. As a first step we determined known vasodilatory mediators in plasma samples obtained from the porcine AMI experiments. Levels of NO, PGI₂ and VIP were higher in APOSEC treated animals compared to control pigs (Figure 13).

Figure 13

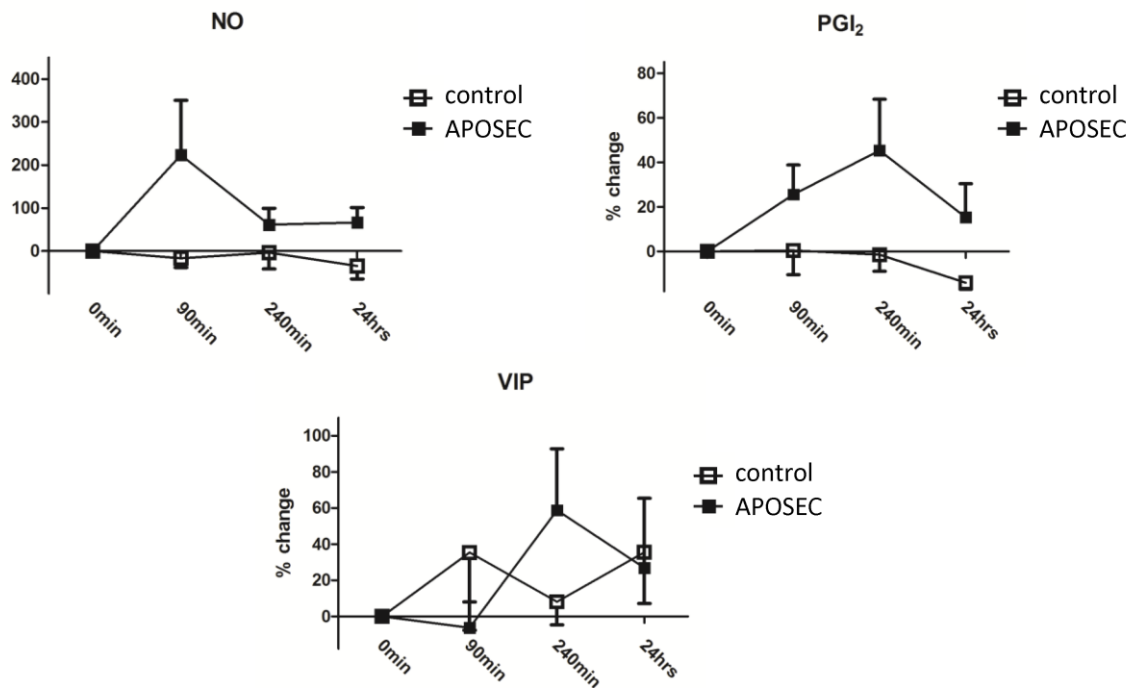


Figure 13 shows systemic levels of vasodilatory mediators in plasma samples obtained after AMI induction. APOSEC treated pigs had higher levels of NO, PGI₂ and VIP when compared to control animals receiving lyophilized medium (n=6-7).

Next we tried to transfer this *in vivo* notion to an *in vitro* experimental setting. Therefore, we treated HUVEC cultures with APOSEC and evaluated its impact on iNOS and phospho-eNOS expression. Interestingly, co-incubation with APOSEC derived from $2.5 \cdot 10^6$ cells resulted in a phosphorylation of the constitutively expressed eNOS within 60min and a *de novo* production of iNOS within 24hrs (Figure 14). This finding could be confirmed when treating isolated coronary arterial rings obtained from hearts of slaughtered pigs with APOSEC.

Figure 14

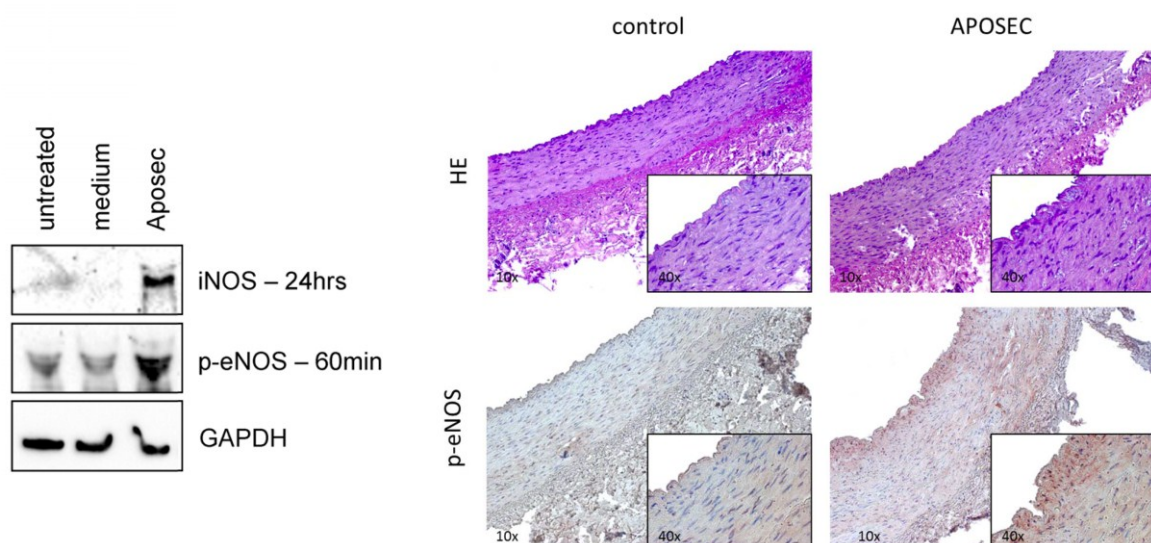
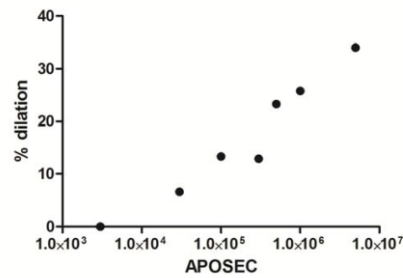


Figure 14 depicts APOSEC induction of iNOS and p-eNOS in HUVEC cultures after 60min or 24hrs, respectively (left, n=3). This finding could be confirmed by immunohistochemical evaluation of isolated coronary rings treated with APOSEC or medium control (right, n=2).

In addition, we evaluated vasomotor effects of APOSEC in myographic experiments. Rings treated with APOSEC showed significant, dose-dependent vasodilation, immediately after adding APOSEC to the experiment (Figure 15). This effect was independent of NOS activity, since blocking NOS by L-NAME could not abrogate the dilation (Figure 16). A possible explanation for this phenomenon could be considerable amounts of NO found in APOSEC preparations ($12 \cdot 10^6$ /mL: 39.5nM; $1.2 \cdot 10^6$ /mL: 16.9nM; $0.12 \cdot 10^6$ /mL: 1.3nM).

Figure 15



Myographic experiments with isolated coronary rings showed a dose-dependent vasodilatory effect of APOSEC (n=7).

Figure 16

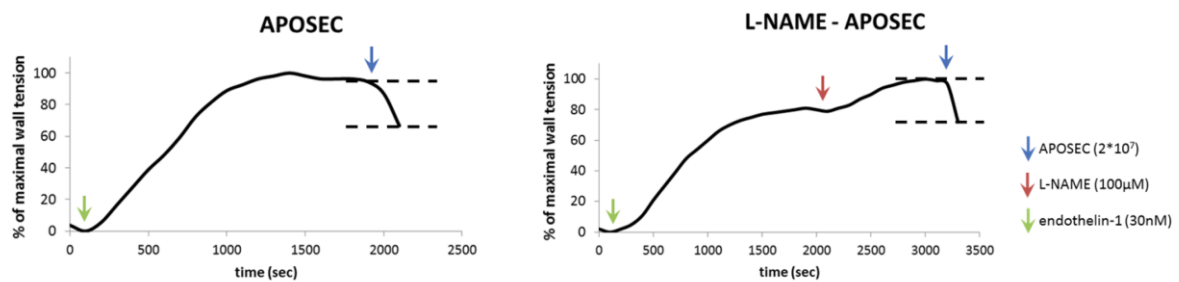


Figure 16 shows that NOS inhibition had no effect on the vasodilatory capacity of APOSEC. Blocking NOS activity by L-NAME could not abrogate the APOSEC mediated reduction of wall tension (n=2).

APOSEC from healthy donors, diabetic patients and CHF patients have comparable features

In a further set of experiments we addressed the question whether beneficial effects of APOSEC are restricted to healthy blood donors. We therefore produced APOSEC from blood donations obtained from insulin-dependent diabetic as well as congestive heart failure patients. Contents of APOSEC from healthy, diabetic (DM) and heart failure (CHF) patients were compared by evaluation of reference cytokines and levels of NO (Figure 17). With the exception of NO, which was moderately higher in APOSEC (DM), no significantly differences were observed throughout the three groups. This finding was proven in two functional assays – platelet aggregation and iNOS/phospho-eNOS induction in HUVEC cultures. Again, no differences between the three conditions were detectable (Figure 18).

Figure 17

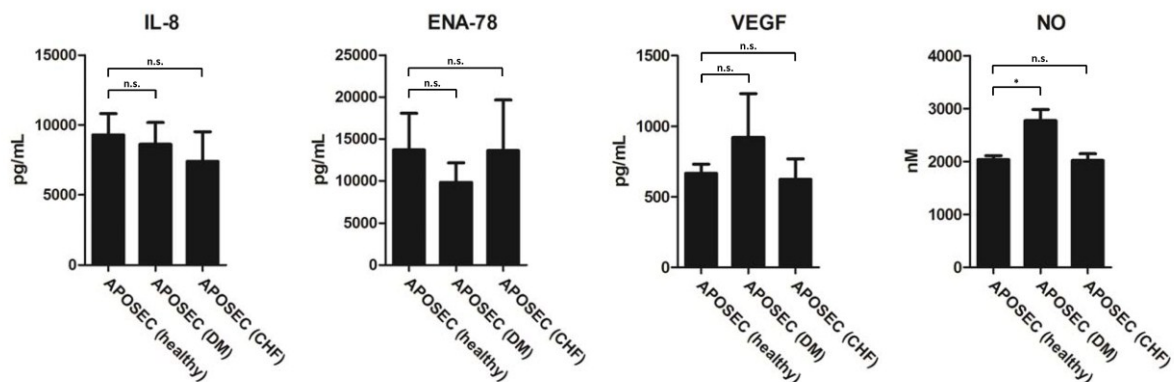
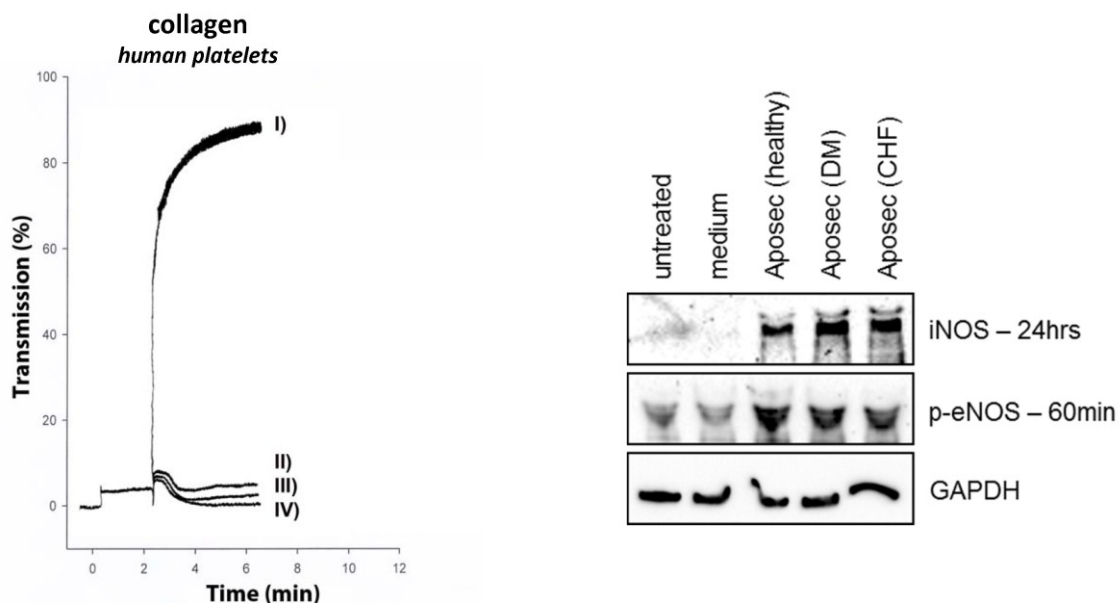


Figure 17 shows the evaluation of three reference cytokines and NO in APOSEC preparation from healthy volunteers, diabetic patients and heart failure patients (n=6-7).

Figure 18



Functional evaluations of different APOSEC preparations are shown in Figure 18. APOSEC (healthy), APOSEC (DM) and APOSEC (CHF) mediated the same anti-aggregatory effects (n=2) and all three compositions induced similar amounts of iNOS and p-eNOS in HUVEC cultures (n=3).

Discussion

Acute myocardial infarction is a major health topic worldwide. More than 3 million people each year are estimated to suffer an acute ST-elevation myocardial infarction, and more than 4 million people experience a non-ST-elevation myocardial infarction per year (41). Over the last decades, establishing early reperfusion of the occluded coronary artery by interventional means or by thrombolytic agents has significantly reduced early mortality. However, successful restoration of the epicardial coronary artery patency does not always lead to an adequate reperfusion at the microvascular level (42). In approximately 40% of patients a lack of perfusion at the microvascular level can be observed (43, 44). This microvascular dysfunction is characterized by an increment of myocyte loss and was shown to be associated with poor clinical outcome (45, 46). In this respect, maintaining or restoring the microvascular blood flow has become a major topic of interest in research. To date, several therapeutic strategies have been suggested: abciximab, adenosine, papaverine, nitroprusside, nicorandil, pexelizumab, the peptide FX06, atrial natriuretic peptide, cyclosporine, intermittent arm ischemia (20, 29).

Since the no-reflow phenomenon is strongly linked to an impaired prognosis, it is important to correctly identify affected individuals. There are several strategies to diagnose a no-reflow (29). In angiographic analysis a reduced TIMI flow and a decreased myocardial blush directly reflect microvascular blood flow impairment (30, 35). ST-segment resolution of <50% as measured by echocardiography is closely linked to MVO (47, 48). In about 25% of patients ST-segment abnormalities persist even though coronary blood flow has been restored. After the initial phase of a myocardial infarction, MVO can be determined by MRI or by myocardial contrast echocardiography using gas-filled microbubbles (49). The MRI is nowadays considered the clinical gold standard for the diagnosis of MVO. During the examination a gadolinium-based contrast agent is injected and delayed phase imaging technique is applied. Since in infarcted myocardium myocytic membranes are ruptured, a rapid distribution of gadolinium chelates into both the intravascular and extracellular space can be observed. In contrast to these hyperenhanced areas, areas of no-reflow lack contrast agent dyeing since the microvasculature is occluded (50).

We have previously evidenced that the secretome of apoptotic PBMC reduces the areas of ischemic damage after AMI. Lichtenauer *et al.* were able to show that apoptotic PBMC and their secretome had an impact on the composition of the extracellular matrix in terms of accumulation of elastic fibres in the border zone between viable myocardium and the post-infarction scar (18). In addition to that, the secretome of apoptotic PBMC was shown to induce phosphorylation of AKT, p42/p44 extracellular signal-regulated kinases (Erk1/2), p38 MAPK, Hsp27, c-Jun and CREB in human primary cultured cardiomyocytes in a dose-dependent fashion. The expression of anti-apoptotic proteins such as Bcl-2 and BAG1 was up-regulated in APOSEC-treated cardiac myocyte cultures (15). This present work extends the cardioprotective effects of APOSEC with findings on MVO. Two aspects of the complex and still elusive pathophysiology of MVO were highlighted in this study: platelets and vasomotor state.

Platelets are an important contributor to MVO during AMI. They are components of microembolisms, frequently occurring in AMI patients (51) and are responsible for occluding microvasculature even in remote regions as previously shown by Barrabes *et al.* (23). Besides this, activated platelets function as inflammatory cells (52). They can quickly release preformed chemotactic factors, which are stored in their alpha-granules (53). Moreover, platelets modulate the inflammatory response of other cells including leukocytes and endothelium by direct cell contact (54, 55). These findings have led to therapeutic applications of blocking agents interacting with platelet activation/aggregation. To date, only abciximab, a monoclonal antibody directed against the fibrinogen receptor GPIIb-IIIa, has been successfully validated in clinical trials (56-60). Thus, current guidelines recommend abciximab in primary PCI for ST-elevated acute coronary syndromes – class IIa recommendation (61).

In this current study we could show that APOSEC inhibits platelet activation and aggregation *in vitro* and *in vivo*. One suggested mechanism responsible for this was an induction of VASP phosphorylation

by APOSEC. VASP plays a key role during the change from a resting discoid state to an activated configuration with the formation of filopodia. This change of configuration is essential to form a stable clot. The phosphorylated form of VASP inhibits this mechanism. Interestingly, in a recent study by Kohler *et al.* the phosphorylation state of VASP was shown to directly correlate with the extent of myocardial ischemia/reperfusion injury. An increase of intra-platelet VASP phosphorylation effectively prevented platelet activation and inhibited platelet-neutrophil complex formation. These findings could be confirmed with VASP knock-out animals and by bone marrow chimeric animals (62). In addition, therapeutic augmentation of phosphorylated VASP using a guanylyl cyclase activator was shown to be effective in a rodent animal model of AMI (63).

The vasomotor state of the coronary artery system is an important feature during MVO (64). Endothelial dysfunction in the small coronary vasculature is a major pathophysiological component of the no-reflow phenomenon (65). During reperfusion the endothelium is injured by oxygen free radicals. This damage leads to impaired endothelium-dependent vasodilation in the small coronary arteries (66). In addition, aspirates from coronary arteries contain vasoconstrictor factors, further contributing to impaired vasodilation (25). The concept of increased vasomotor tone in the area of MVO is supported by several clinical trials, testing different vasodilators during PCI. To date, the most promising agent is adenosine. A first trial performed by Testa and colleagues evaluated the role of adenosine during AMI (67). 54 patients were enrolled in this placebo-controlled trial. 27 patients received an intracoronary adenosine injection during PCI and the remaining 27 patients were treated with a saline injection. An intracoronary administration of adenosine was shown to improve microvascular perfusion leading to an improved clinical course after PCI. However, these promising results could not be confirmed by larger studies evaluating an intravenous application of adenosine (68). Nicorandil is a mitochondrial potassium channel opener with a nitrate compound and is a potent vasodilator. Intravenous administration of nicorandil led to an improvement in microvascular perfusion, infarct size, and clinical outcomes (69, 70). Intracoronary administration of "classical" vasodilators e.g. nitroprusside or verapamil led to significant improvements in coronary flow in clinical trials (71, 72). Currently, adenosine, verapamil or nitroprusside are recommended for the treatment of no-reflow (28).

The data on vasodilation of this work can be divided into two different mechanisms. A direct, short term effect, as determined by the coronary ring assays, seems to be mediated by considerable amounts of NO found in APOSEC preparations. Since during the production process APOSEC is thoroughly dialyzed the possibility that NO decomposition products are present in APOSEC can be excluded. Protein adducts of nitric oxide may represent the biologically active ingredient of APOSEC regarding direct vasodilatory effects. Intermediate vasodilatory effects following APOSEC treatment are linked to phospho-eNOS and iNOS induction. This finding is essential, since NOS have been previously shown to contribute to cardioprotection (73, 74).

An interesting aspect was added to this study by the evaluation of APOSEC produced from diseased patients. Levels of reference cytokines as well as the impact on two functional assays were comparable in APOSEC from healthy, diabetic and CHF patients. This is an important finding, because it extends a possible future allogeneic use to an autologous application. Donor safety, which is a continuous issue for "biologicals", could be bypassed by producing APOSEC in an autologous setting.

PART II – SECRETOME FROM MONONUCLEAR CELLS ATTENUATES MYOCARDITIS IN AN EXPERIMENTAL AUTOIMMUNE MYOCARDITIS MODEL

Introduction

Myocarditis – an autoimmune disease?

Myocarditis is a disease entity characterized by an inflammation of the heart muscle. Since most cases of this illness have a subclinical course, epidemiological data can only be assumed from post-mortem studies. Myocardial inflammation can be found in 1 to 9 percent of all routine autopsies (75, 76). The clinical presentation of a myocarditis is highly variable – symptoms range from asymptomatic presentations with subclinical echocardiographic abnormalities to fulminant heart failures with acute decompensation (77).

The cause of myocarditis in the individual patient often remains unknown. However, studies have implicated a broad range of microorganisms to be associated with the development of the disease including viruses, bacteria, protozoa and even parasites. It is generally believed that in most cases a viral infection is the initiating factor of a myocarditis. Several studies have pointed out the involvement of cardiotropic viruses, primarily enteroviruses, in the disease. Increased antibody titers against viral components can be frequently found in the serum of acute myocarditis patients (78). More recently viral ribonucleic acid (RNA) could be detected in myocardium of patients suffering from idiopathic dilated cardiomyopathy (79, 80). The current understanding of the etiology of myocarditis mostly comes from animal models. In these models susceptible strains are infected with a cardiotropic virus, e.g. encephalomyocarditis virus or coxsackievirus B (81). This leads to a mononuclear infiltrate of the heart (NK cells, B cells, T cells) and in most cases viral clearing. However, a chronic phase can develop, which is characterized by the absence of virus but continuing inflammation. During this chronic phase autoimmunity is believed to play an important role (81).

The autoimmune hypothesis of myocarditis is corroborated by observations of auto-reactive, cytotoxic T cells as well as increased serum auto-antibodies in patients (82, 83). Furthermore, an auto-reactive, mononuclear myocardial infiltrate has been described in post-mortem examinations corroborating autoimmunity in the pathogenesis of myocarditis (75).

Stem cell therapy in inflammatory diseases

The idea to use stem cells to treat inflammatory diseases is based on *in vitro* observations of immunomodulatory features of those cells. In co-culture experiments with activated lymphocytes mesenchymal stem cells can suppress lymphocytic proliferation in a dose-dependent manner (84-86). Additionally, co-culturing experiments of mesenchymal stem cells with other cell populations of the immune system resulted in an altered cytokine secretion profile of dendritic cells (DC), T cells (T helper 1 and 2), and natural killer (NK) cells (87). The presence of mesenchymal stem cells predominantly led to an induction of an anti-inflammatory, tolerance inducing milieu in these experiments (summarized in Figure 19). Stem cells have been shown to mediate their immunomodulating effects both via direct cell–cell interactions as well as via secretion of soluble paracrine factors (88).

Figure 19

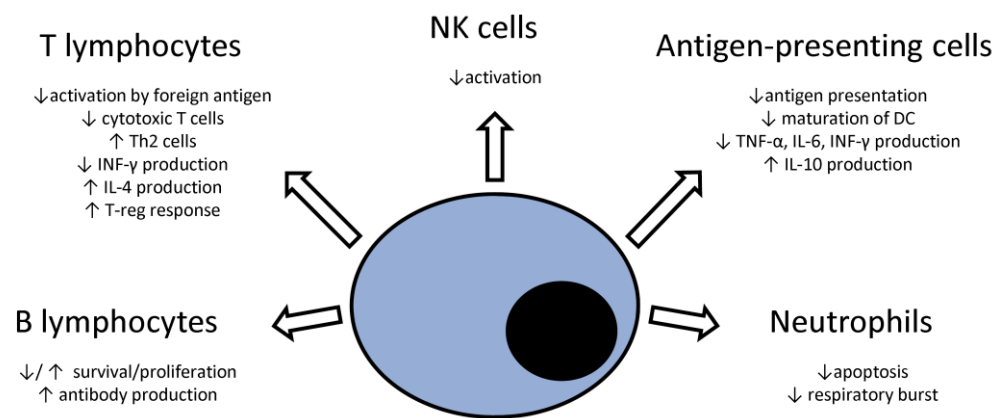


Figure 19 summarizes various effects of stem cells on different immune cells; adapted from (88).

The *in vitro* observed immunomodulatory features of stem cells led to experiments in the field of transplant research. Mesenchymal stem cells induced tolerance in heart transplantation, islet transplantation and in skin transplantation models (86, 89, 90). Regarding classical autoimmune diseases stem cells already have proven beneficial in graft-versus-host disease, diabetes, rheumatoid arthritis and inflammatory bowel diseases (91-95). Stem cell therapy has also been evaluated in the setting of myocarditis. Transplantation of mesenchymal stem cells attenuated the inflammatory infiltrate and ameliorated functional parameters in a rat myocarditis model (96, 97).

Stem cell conditioned medium for therapeutic use in inflammatory diseases

Since stem cells have been proven beneficial in treating autoimmune diseases and *in vitro* data clearly state that some of their main immunomodulatory features are mediated through paracrine mechanisms, the next logical step would be to test conditioned medium as a therapeutic agent in inflammatory disorders. However, in contrast to ischemic diseases only sparse data on this subject are available in the literature. In a work by Cargnoni *et al.* bleomycin-induced fibrosis could be effectively treated by an intrapulmonary injection of conditioned medium generated from human amniotic mesenchymal tissue cells – serving as a stem cell source (98). In a study by Ohnishi *et al.* the development of an autoimmune myocarditis was inhibited by injection of mesenchymal stem cells. In some supplementary experiments stem cell conditioned medium was tested and could ameliorate myocardial damage (97).

Similarities of secretome derived from stem cells and peripheral blood leukocytes

The secretome of stem cells comprises of a myriad of components. Proteomic analysis revealed that a broad repertoire of trophic and immunomodulatory cytokines are produced by stem cells (99, 100). Interestingly, the pattern of secreted proteins is consistent throughout different stem cell subgroups (10). In addition to that Korf-Klingebiel *et al.* have shown that the secretome of bone marrow stem cells and the secretome of peripheral blood leukocytes are in many respects comparable. They could evidence that the secreted proteins are more or less similar in both cell preparations leading to uniform results in functional assays e.g. endothelial cell proliferation, migration, tube formation and cell sprouting in a mouse aortic ring experiment (14). Based on these observations our research group started to experiment with secretomes derived from peripheral blood mononuclear cells. We were able to show that the secretome of apoptotic, irradiated PBMC could ameliorate the ischemic damage following an AMI (15). As a possible mechanism a modulation of the inflammatory reaction

following the ischemic event was suggested (17). In a rat AMI model lower levels of S100b+ dendritic cells were found in animals receiving viable PBMC or apoptotic PBMC when compared to control rats (Figure 20).

Figure 20

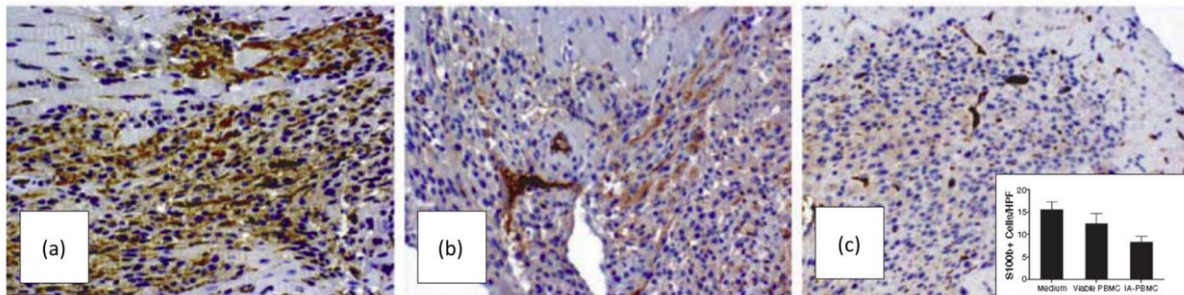


Figure 20 depicts expression of S100b+ dendritic cells. AMI rats treated with control medium (a) had dense clusters of antigen presenting cells whereas the inflammatory response was abrogated by the injection of viable (b) or apoptotic (c) PBMC solutions (cells and secretome – adapted from (17)).

The EAM model – role of CD4+ cells

The experimental autoimmune model (EAM) of the mouse was first described by Pommerer *et al.* (101). The experimental basis of the EAM model is an immunization of a susceptible mouse strain (e.g. Balb/c) with a cardiac specific peptide – MyHC- $\alpha_{614-634}$. After a twofold subcutaneously injection of a homologous α -myosin-fragment (MyHC- $\alpha_{614-634}$ Ac-SLKLMATLFSTYASAD-OH) together with a strong adjuvant (complete Freund adjuvant +/- pertussis toxin) mice develop a severe but self-limited myocarditis (101, 102). The course of the disease can be outlined as follows: day 14 (initiation of inflammation), day 17 (early inflammation), day 21 (climax of inflammation), day 35 (reconvalescence) and day 60 (total recovery). In contrast to the “classical” way of myocarditis induction by cardiotropic viruses, which leads to myocardial infiltrations in only a minority of cases, the EAM method results in the development of myocarditis in nearly 100% of animals. The myocardial infiltrate in the EAM model is dependent on CD4+ cell function (103). Depletion of CD4+ cells totally abrogates the induction of myocarditis in this model (104-106). The EAM model is currently considered the best available animal model mimicking the chronic autoimmunity-dominated phase of myocarditis. The main advantage of this model is the possibility to study pathophysiological processes and treatment effects without the use of an infective agent (107).

Apoptosis

Apoptosis is a basic process during which cells die strictly orchestrated, the so called “programmed cell death”. It is a common feature under normal physiological conditions but is also a frequent finding in a broad range of diseases.

In contrast to necrotic cell death, which is typically caused by exposure to a noxious agent, programmed cell death is dependent on the initiation of caspases, which leads to endonuclease activation with the consequence of DNA fragmentation and liberation of histones. Apoptosis can be induced by two divergent pathways: an exogenous pathway, mediated through death receptors, and an intrinsic pathway, also termed mitochondrial-mediated pathway (108, 109).

The exogenous pathway is usually activated by Fas, TNF receptor type 1, death receptor 3, TRAIL receptor type 1 or 2. Upon triggering, Fas-associated death domain (FADD) recruits caspase 8, which initiates the apoptotic proteolytic cascade. The mitochondrial pathway is initiated by a myriad of stress stimuli on the cell e.g. reactive oxygen species. It is characterized by a well-balanced interplay of proapoptotic (Bcl-2) and anti-apoptotic factors (Bcl-2). The mitochondrial pathway

results in an initial activation of caspase 9 (109). Both, active caspases 8 (extrinsic) and active caspase 9 (intrinsic) induce activation of caspase 3, which is in the final common cell death pathway.

Detection of apoptosis is nowadays simple, since many, highly standardized, assays are commercially available. Apoptotic cells can either be recognized by simple microscopic analysis. Apoptotic cells look shrunk, a deformation of the cell membrane and condensation of the nuclear chromatin can be found. For cells in suspension a flow-cytometric analysis of apoptosis by Annexin V is nowadays the gold standard. Early after the initiation of apoptosis, cells lose their phosphatidylserine (PS), which is – under normal conditions – actively kept on the inner surface of the cell membrane, to the outer cell surface. This translocated PS can be easily detected a fluorochrome-conjugate of Annexin V, a protein that can bind to PS.

In regard to autoimmune diseases apoptosis induction of auto-reactive effector cells is one possible therapeutic option. The application of apoptosis-inducing antibodies has been proven effective in the treatment of myocarditis (104-106, 110, 111).

Aim of the study

The aim of the study was to evaluate the impact of a single high-dose application of MNC secretome on the development of myocarditis in the EAM model. We have shown in previous studies that MNC secretome can be easily and safely applied in models of acute myocardial infarction. Since the inflammatory reaction following the ischemic event could be effectively blocked in those studies, we aimed to further characterize possible immunomodulatory features of MNC secretome. As the EAM model is dependent on CD4⁺ cell function, we further planned to evaluate the effects of MNC secretome on highly purified CD4⁺ cells *in vitro*.

Material and Methods

Generation of murine and human MNC secretome

The standardized production protocol of MNC secretome, as developed in our laboratory (15), had to be adapted for mouse experiments, since processing peripheral blood samples did not lead to sufficient numbers of mononuclear cells. Thus, splenocytes were used instead of PBMC. Spleens were removed from 10 donor Balb/c mice under sterile conditions (112). Single cell suspensions were generated by removing connective tissue and debris using a 70 μ m and subsequently a 40 μ m cell strainer (BD, NJ, USA). After lysing the erythrocytes (Sigma Aldrich, MO, USA), splenocytes were resuspended in UltraCulture serum-free medium (Cambrex Corp., North Brunswick, NJ, USA; 1×10^6 cells/ml). Cells were incubated for 24hrs in a humidified atmosphere (5% CO₂ and 37°C) and the thereby conditioned medium was dialyzed against ammonium acetate (at a concentration of 50mM, cut-off 3.5kD), sterile filtered, frozen, lyophilized and kept at -80°C until further used. MNC secretome pooled from ten different donor mice was used for the experiments. For *in vitro* experiments PBMC were obtained from young healthy volunteers (ethics committee vote: EK-Nr 2010/034). MNC were purified from venous whole blood samples by means of Ficoll-density gradient centrifugation. All other production steps were similar to the protocol described above. Dialyzed and lyophilized control medium served as a negative control.

EAM model

All animal experiments were performed at the University of Vienna, Core unit for Biomedical Research, with a positive vote from the animal experiment committee (vote:66.009/0055-II/10b/2010). Experiments were performed in accordance to the Guide for the Care and Use of Laboratory Animals by the National Institutes of Health (NIH Publication No. 85-23). EAM was induced in 6-8 week-old Balb/c mice by subcutaneous injection of 150 μ g of the MyHC- α (MyHC- $\alpha_{614-634}$: Ac-SLKLMATLFSTYASAD-OH) or ovalbumin emulsified 1:1 in PBS/CFA (1mg/ml, H37Ra) with a 7-day interval between injections (113). Secretome of 4×10^6 or 10×10^6 syngeneic MNC cultures was intraperitoneally injected at different time points (day 0, day 7, day 14). Injections of lyophilized unconditioned culture medium served as negative control. Mice were sacrificed on day 21 (climax of inflammation) and hearts were evaluated for myocardial infiltrates.

Myocarditis grading

Myocardial infiltrates were quantified on the basis of hematoxylin-eosin stains. Myocardium affected by myocarditis was defined by regions showing myocardial hemorrhage, infiltration by inflammatory cells, and myocardial necrosis. The infiltrates were scored in a blinded way by a semiquantitative scale (0 indicated no inflammatory infiltrates; 1: small foci of inflammatory cells between myocytes; 2: larger foci of >100 inflammatory cells; 3: <10% of a cross-section involved; 4: >30% of a cross-section involved), as previously described (114).

Enzyme-linked immunosorbent assays

Levels of circulating IL-1 β , IL-6 and TNF- α in plasma samples obtained from mice on day 21 were determined by commercially available ELISA kits (R&D Systems, Minneapolis, MN, USA) following the manufacturer's instructions. Content of sFAS, sFASL, sCD40, and sCD40L in MNC preparations was also measured by means of ELISA (R&D Systems, Minneapolis, MN, USA). Auto-antibody formation against MyHC- $\alpha_{614-634}$ was quantified by a solid phase ELISA. Plates were coated overnight with 5 μ g/ml MyHC- $\alpha_{614-634}$ dissolved in bicarbonate buffer. Plasma obtained from mice at day 21 was diluted 1:10 for IgM, 1:50 for IgG1, 1:10 for IgG2a and IgG2b, and 1:50 for IgG3 measurement. After incubating diluted plasma for 24hrs at 4°C monoclonal rat anti-mouse IgM, IgG1, IgG2a, IgG2b and

IgG3 antibodies (Pharmingen, CA, USA) diluted 1:1000 were used as primary antibodies. A HRP-conjugated goat anti-rat antiserum (Amersham, Biosciences, UK) diluted 1:2000 together with ABTS (60mM/l citric acid, 77mM/l $\text{Na}_2\text{HPO}_4 \times 2\text{H}_2\text{O}$, 1.7mM/l ABTS (Sigma, MO, USA), 3mM/l H_2O_2) was used as the detection system.

Immunostatus

Whole blood samples were obtained 12hrs and 36hrs after mice were treated with MNC secretome or medium control (day 14). Erythrocytes were lysed (Sigma, MO, USA) and cell pellets were resuspended in FACS buffer (PBS, 0.1% bovine albumin, 0.1% sodium acid). Cells were stained with fluorochrome conjugated anti-CD4, anti-CD8 (both Acris, Herford, Germany) and 7-Aminoactinomycin D (7-AAD; Beckman Coulter, CA, USA). Percentages of CD4+ cells, CD8+ cells, the CD4+/CD8+ ratio and percentages of 7-AAD/CD4+ double positive cells were determined by flow cytometry (Beckman Coulter, CA, USA).

Splenocyte proliferation assay

EAM mice were treated with MNC secretome or control medium at day 14. After sacrifice at day 21, spleens were homogenized, as described above, and splenocytes (1×10^5) were co-cultured in a humidified atmosphere (5% CO_2 and 37°C) with different concentrations of MyHC- $\alpha_{614-634}$. After five days cells were pulsed for 18hrs with ^3H -thymidine (3.7×10^4 Bq/well; Amersham Pharmacia Biotech, Uppsala, Sweden). Splenocytes were harvested and ^3H -thymidine incorporation was measured in a liquid scintillation counter.

CD4+ cell proliferation assay

PBMC were obtained from blood draws of healthy volunteers by Ficoll density centrifugation. CD4+ cells were purified by negative selection using a MACS no-touch CD4+ purification kit (Miltenyi Biotec, Bergisch Gladbach, Germany). 1×10^5 highly purified CD4+ cells were either stimulated with phytohemagglutinin ($7 \mu\text{g/ml}$, Sigma, MO, USA) or a monoclonal antibody against CD3 ($10 \mu\text{g/ml}$, Becton Dickinson, NJ, USA). MNC secretome or control medium was added in different concentrations. Plates were incubated for 5 days, pulsed for 18hrs with ^3H -thymidine and the proliferative response was measured as described above.

Detection of apoptosis

Purified human CD4+ T-cells or JURKAT cells (ATCC, VA, USA) were incubated at a concentration of 1×10^6 in a humidified atmosphere. MNC secretome of 1.1×10^6 cells or control medium was added and AnnexinV-fluorescein/propidium iodide (FITC/PI) stains (Becton Dickinson, Franklin Lakes, NJ, USA) were performed at different time points (0hrs, 6hrs, 12hrs, 24hrs). In a second set of experiments apoptosis was monitored by determination of released histones 18hrs after adding MNC secretome. Histone-associated DNA fragments (mono- and oligonucleosomes) were determined using a commercially available kit (Roche Molecular Biochemicals, Penzberg, Germany). For blocking experiments CD4+ cells were pre-incubated with different caspase inhibitors (Z-VAD, Z-DEDV, Z-IETD, Z-LEHD; purchased from R&D, MN, USA) for 30min. Then MNC secretome or control medium was added. In some experiments neutralizing anti-CD40L, anti-TRAIL1, anti-TRAIL2, anti-FASL, anti-VEGF, anti-IL8, anti-ENA78, anti-MMP9 or anti-isotype antibodies were added.

Statistical analysis

Results are depicted as means \pm standard error of the mean and levels of significances were determined by student's t-test. Data analysis was performed with SPSS 18.0 (SPSS inc., United

States). A P-value less than 0.05 was regarded as statistically significant (* indicates $p < 0.05$; ** indicates $p < 0.01$; *** indicates $p < 0.001$).

Results

MNC secretome attenuates myocardial infiltrate

Based on the primary hypothesis of this study we tested the impact of MNC secretome on the development of myocarditis in the EAM model. Since no data exist on the optimal time point of treatment using immunomodulatory conditioned medium, we injected MNC secretome and control medium at different time points. An intraperitoneal application of MNC secretome during sensitization (day 0 or day 7) had no impact on the development of a myocardial infiltrate (day 0: MNC secretome 2.8 ± 0.6 ; control medium: 2.3 ± 0.6 ; $p=0.606$; day 7: MNC secretome 3.1 ± 0.3 ; control medium: 3.4 ± 0.5 ; $p=0.638$), although the application of a high-dose therapy at day 7 seemed to have a beneficial effect (Figure 21).

Figure 21

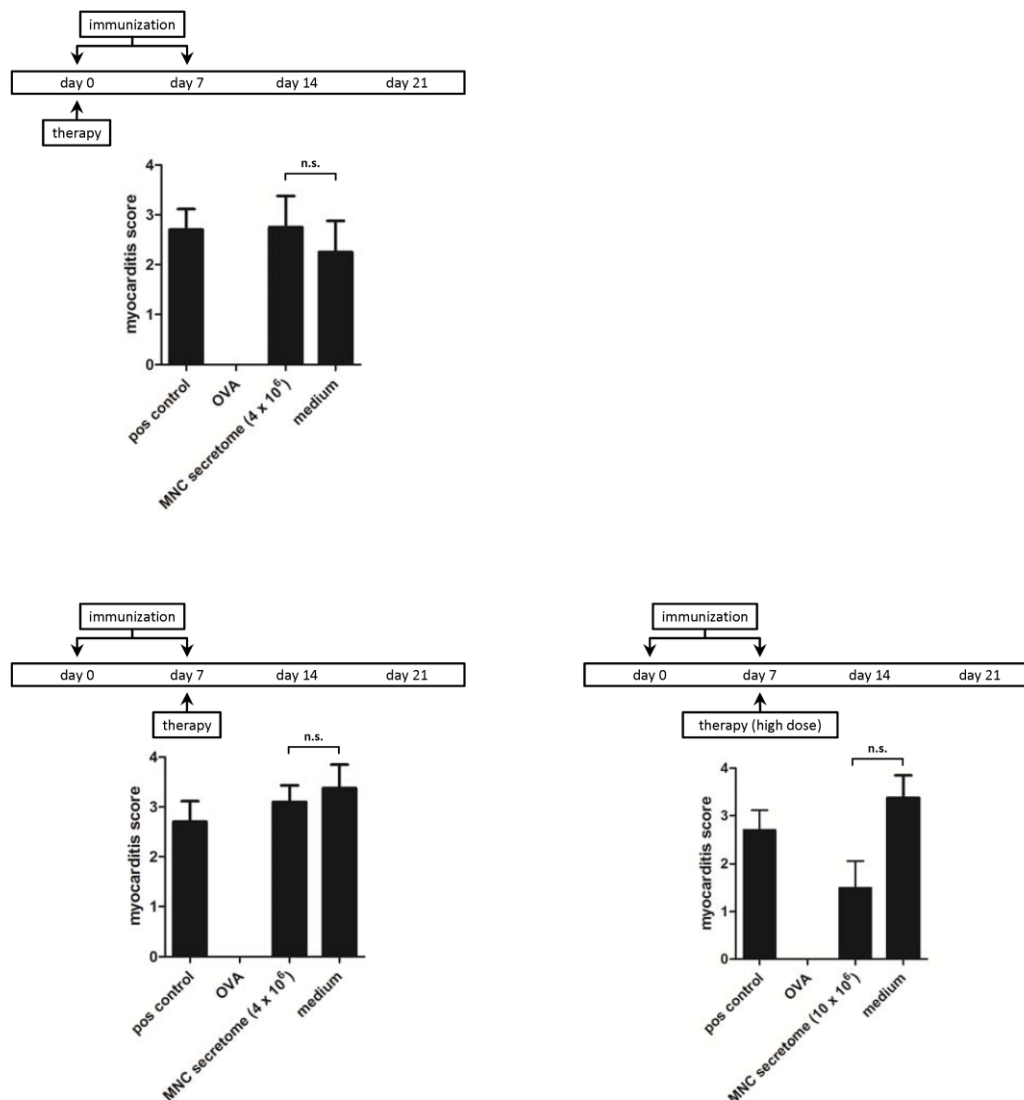
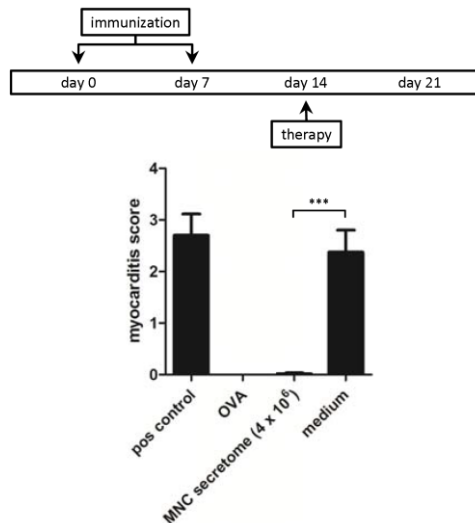


Figure 21 depicts myocarditis scores of explanted hearts. MNC secretome applied during the sensitization process (day 0 and day 7) had no impact on the development of myocarditis ($n=4-10$).

However, an injection of MNC secretome on day 14 resulted in a significant reduction of the myocarditis score (MNC secretome: 0.1 ± 0.0 ; control medium: 2.4 ± 0.4 ; $p=0.0001$; Figure 22).

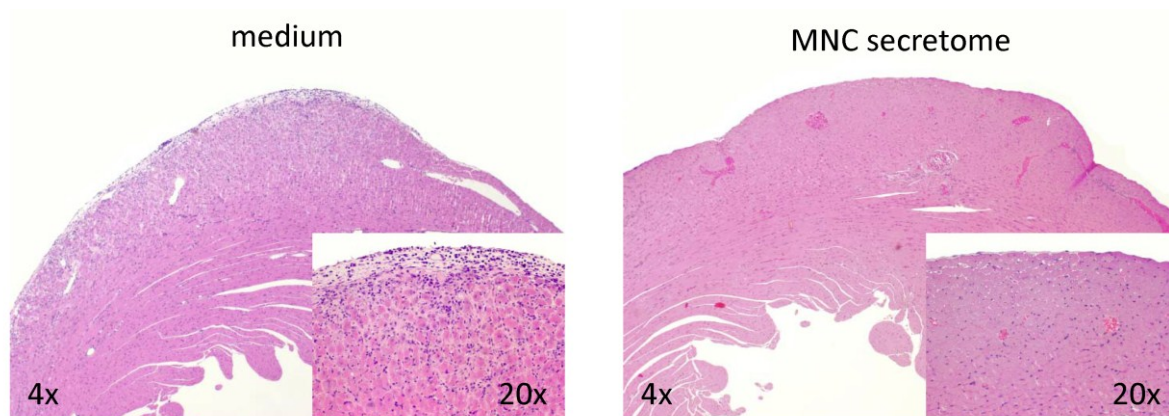
Figure 22



Histological evaluations of mice treated on day 14 are depicted in Figure 22. The myocarditis score was significantly lower in mice treated with MNC secretome during a full blown myocarditis when compared to untreated or medium treated animals (MNC secretome: 0.1 ± 0.0 ; control medium: 2.4 ± 0.4 ; $p=0.0001$; $n=4-10$).

A thorough histological work-up revealed dense lymphocytic infiltrates in the hearts of control mice, whereas only sparse areas of inflammation and necrosis could be found in MNC secretome treated animals, as depicted in Figure 23.

Figure 23



Two representative H&E stainings are shown in Figure 23. A medium treated animal evidenced a dense lymphocytic infiltrate whereas the MNC secretome treated mouse had a more or less unaffected myocardium.

Systemic inflammatory reaction is dampened by MNC secretome

In a next step we analyzed plasma samples obtained on day 21 for inflammatory mediators (TNF α , IL-1 β and IL-6). In all of our samples levels of TNF α were below the detection limit of the ELISA kit. Levels of IL-1 β and IL-6 were lower in MNC treated animals when compared to controls (Figure 24).

Figure 24

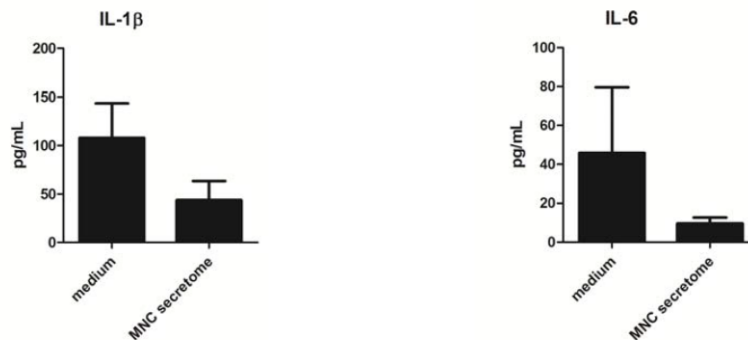
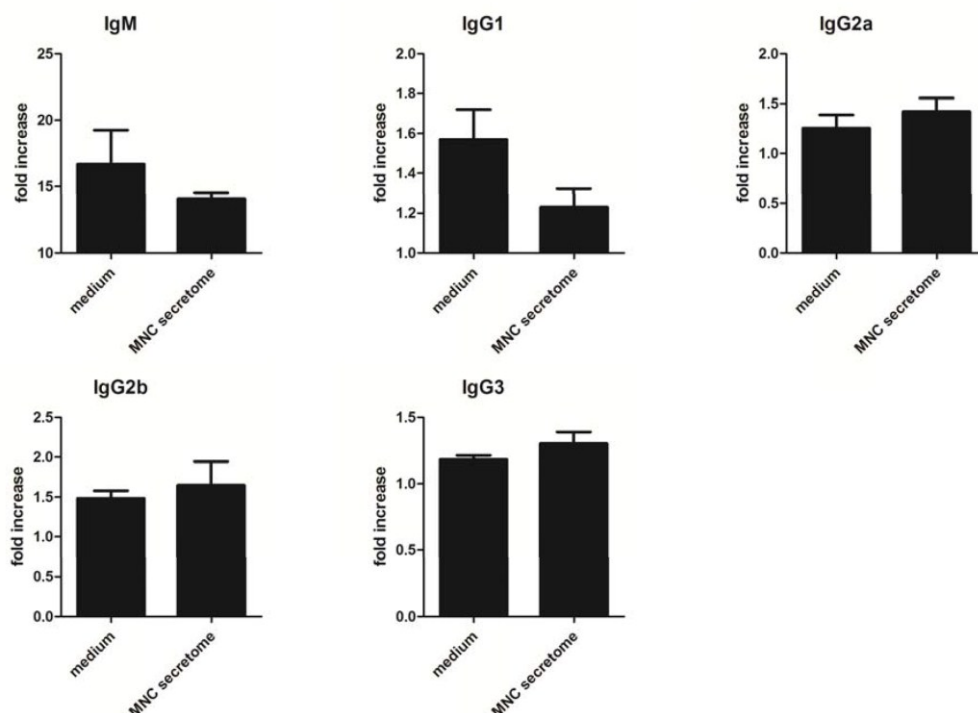


Figure 24 depicts systemic levels of inflammation. IL-1 β and IL-6 were lower in plasma samples obtained from MNC treated mice when compared to untreated animals (IL-1 β : 107.9 \pm 35.5 vs. 43.7 \pm 19.5; IL-6: 45.9 \pm 33.7 vs. 9.6 \pm 3; n=6).

Auto-antibody formation is a common feature during myocarditis and has been previously described in the EAM model (114). We therefore determined levels of anti-MyHC- $\alpha_{614-634}$ antibodies in plasma samples obtained on day 21. Immunoglobulin subclasses were analyzed by means of a solid-phase ELISA. Auto-antibody formation could be observed in both groups (MNC secretome treated and medium controls). However, lower levels of IgM and IgG1 were found in treated animals than in control animals, although, these differences did not reach significance. Levels of IgG2a, IgG2b and IgG3 against MyHC- $\alpha_{614-634}$ were similar in both groups (Figure 25).

Figure 25



Levels of auto-antibody formation are shown in Figure 25. MNC treated mice had lower levels of IgM and IgG1. Levels of IgG2a, IgG2b and IgG3 were unaffected by the treatment (n=6).

Splenocyte proliferation to MyHC- $\alpha_{614-634}$ is impaired in MNC secretome treated animals

Previous studies have shown that the proliferative response of splenocytes to the myosin peptide is strongly linked to the development of a myocardial infiltrate in the EAM model (114, 115). Spleens were obtained on day 21 from animals treated with control medium or MNC secretome. Splenocytes were re-stimulated *in vitro* with different concentrations of MyHC- $\alpha_{614-634}$. The proliferative response to the peptide was significantly impaired in splenocytes from mice treated with MNC secretome when compared to splenocytes obtained from control mice (Figure 26).

Figure 26

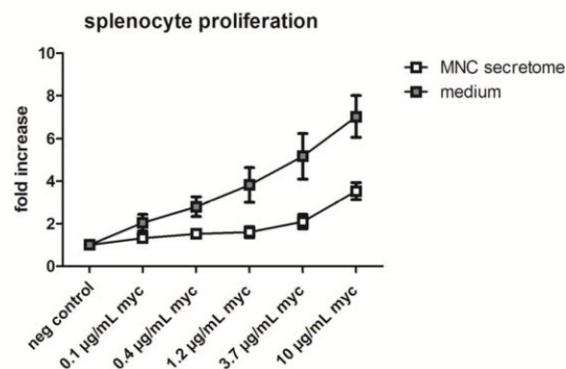


Figure 26 shows proliferation experiments. Splenocytes treated with MNC secretome had a reduced proliferative capacity when stimulated with different concentrations of MyHC- $\alpha_{614-634}$. Control splenocytes obtained from untreated mice showed a strong proliferative response to the peptide (n=5-6).

Anti-proliferative effects of MNC secretome on CD4+ cells

The development of a myocardial infiltrate is strongly linked to CD4+ cell function in the EAM model. Therefore, the effect of MNC secretome on CD4+ cells was further evaluated in *in vitro* experiments. CD4+ cells were purified from healthy volunteers and the effect of MNC secretome on their proliferative capacity was evaluated by incorporation of ^3H -thymidine. CD4+ cell proliferation was dose-dependently inhibited by treating cells with MNC secretome in PHA and anti-CD3 induced proliferations (Figure 27).

Figure 27

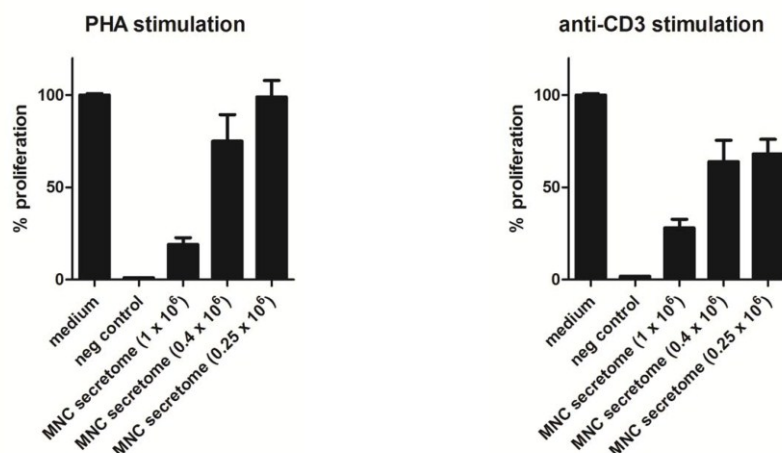


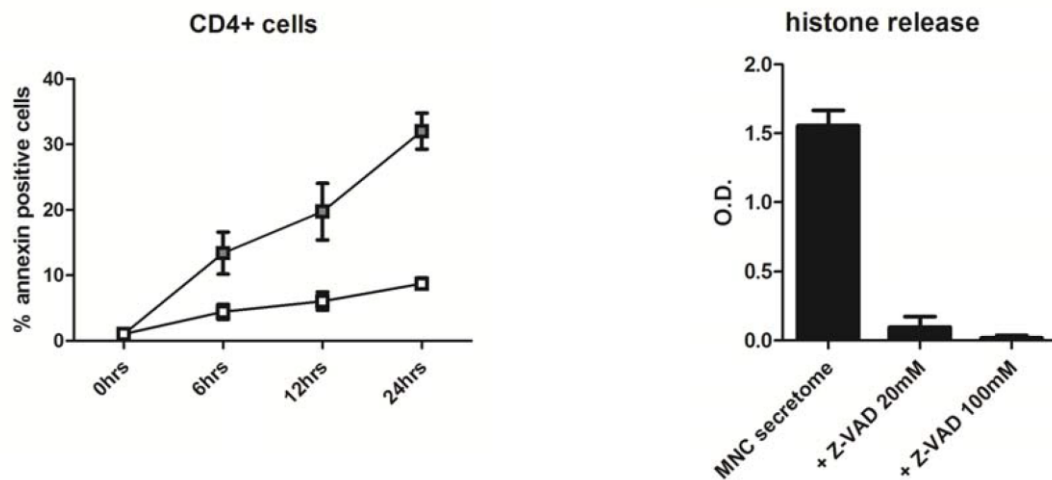
Figure 27 depicts stimulation experiments of purified human CD4+ cells. Cells treated with MNC secretome had a significantly impaired proliferative response to PHA and anti-CD3. These effects were observed in a dose-dependent manner (n=5).

Impact of MNC secretome on viability of CD4+ and JURKAT cells

We evaluated the impact of MNC secretome on cell viability by means of flow cytometry and measurements of histone release. Purified CD4+ cells were incubated with the compound and Annexin V/PI stains were performed. MNC secretome induced apoptosis in CD4+ cells and JURKAT cells (Figure 28). To prove that this was a specific apoptotic and not a necrotic process due to possible toxic components of the secretome, we added a pan-caspase inhibitor to the experiments. Histone-release was completely blocked by pre-incubation of cells with Z-VAD for 30min (n=2).

Figure 28

human CD4+ cells



JURKAT cells

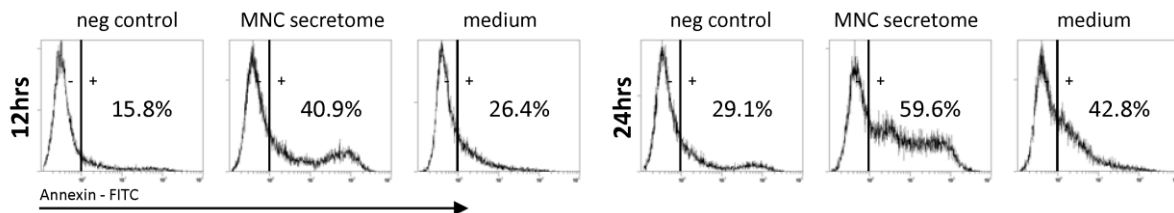
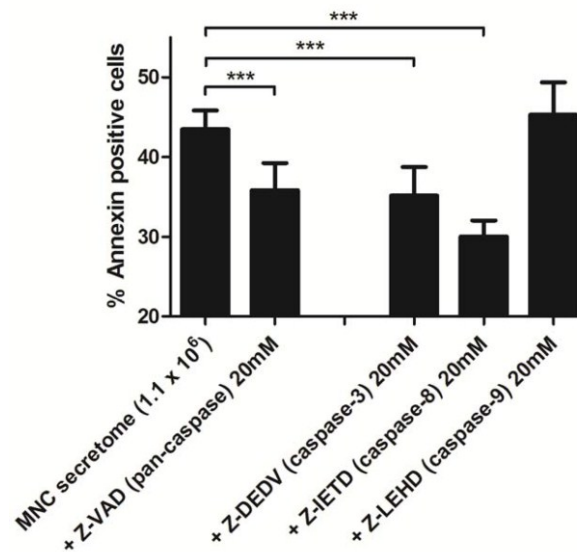


Figure 28 summarizes the apoptotic inducing capacity of MNC secretome. Co-incubation of purified human CD4+ cells and JURKAT cells resulted in an induction of programmed cell death. This was shown by flow cytometric analysis and histone release assays (n=2-8).

Caspase blocking experiments

To define whether the induction of apoptosis is mediated by the external or the mitochondrial pathway, caspase-9, caspase-8 and caspase-3 were selectively blocked by commercially available caspase inhibitors. Pre-incubation of purified CD4+ T cells with caspase-8 and caspase-3 but not caspase-9 resulted in a significant reduction of Annexin positivity. These findings suggest that the external pathway is involved in MNC secretome-mediated apoptosis (Figure 29).

Figure 29



Results of caspase blocking experiments are depicted in Figure 29. Pre-incubation of CD4⁺ cells with a pan-caspase, a caspase-3 and a caspase-8 inhibitor could abrogate the proapoptotic effects of MNC secretome, whereas caspase-9 blocking was ineffective (n=8).

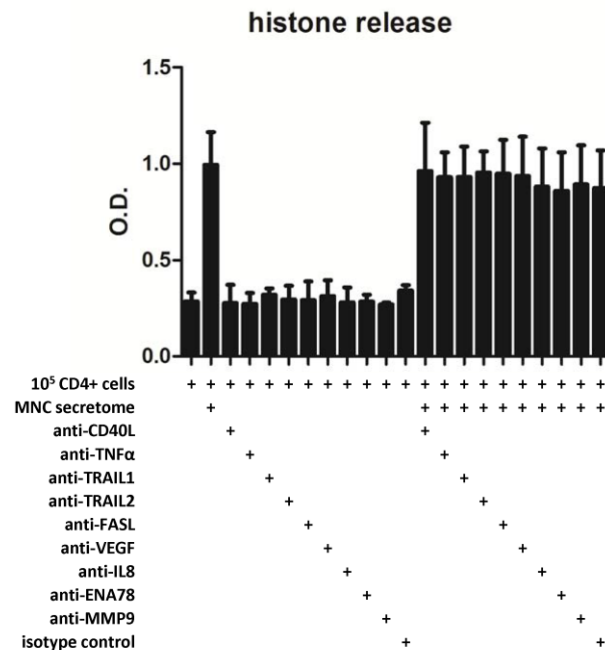
Evaluation of the MNC secretome for known apoptosis inducing factors

Next we sought to determine known apoptosis-inducing factors in the MNC secretome. Interestingly, only low concentrations of TNF- α , sCD40L, sFAS were present in the compound. Levels of sFASL were below the detection limit of the utilized ELISA system (Table 5). In addition, purified CD4⁺ cells co-incubated with blocking antibodies directed against CD40L, TRAIL1, TRAIL2, FASL, VEGF, IL8, ENA78 and MMP9 did not result in a reduced histone release after MNC treatment (Figure 30). Therefore, the pro-apoptotic capacity of MNC secretome must be mediated by (a) still unknown factor(s).

Table 5

	MNC secretome
TNF- α (pg/mL)	6.6 \pm 2.1
sCD40L (pg/mL)	288.1 \pm 101.4
sFAS (pg/mL)	25.4 \pm 18.2
sFASL (pg/mL)	n.d.

Table 5 summarizes results from evaluations of known apoptosis inducing factors in MNC secretome. Only low concentrations of TNF- α , sCD40L, sFAS were present in the compound (n=4).

Figure 30


The effect of using various blocking antibodies on the histone release of purified CD4+ cells is shown in Figure 30. Blocking of CD40L, TRAIL1, TRAIL2, FASL, VEGF, IL8, ENA78 and MMP9 had no impact on MNC secretome mediated apoptosis induction (n=4).

CD4+/CD8+ cell ratio is reduced in MNC secretome treated animals

Based on the *in vitro* findings showing that MNC secretome induced apoptosis in purified CD4+ cells, we performed a small immunostatus in EAM mice. We measured CD4+ and CD8+ cells 12hrs and 36hrs after treating animals with MNC secretome or control medium. The CD4+/CD8+ ratio was reduced in mice receiving the compound when compared to control animals (12hrs: 2.1±0.3 vs. 1.7±0.4; 36hrs: 2.9±0.2 vs. 2.0±0.1). Additionally, numbers of 7-AAD positive CD4+ cells were heightened in MNC secretome treated animals when compared to the control group (Table 6).

Table 6

12hrs	CD4+ (%)	CD8+ (%)	CD4/CD8 ration	CD4+/7-AAD pos
medium	19.9±1.3	10.8±1.9	2.1±0.3	8.1±0.7
MNC secretome	13.3±1.0	9.3±2.0	1.7±0.4	8.8±2.8

36hrs	CD4+ (%)	CD8+ (%)	CD4/CD8 ration	CD4+/7-AAD pos
medium	23.9±1.6	7.4±1.1	2.9±0.2	5.4±0.5
MNC secretome	19.1±2.4	9. ±30.5	2.0±0.1	10.6±1.1

Table 6 shows flow cytometric analysis of blood samples obtained from EAM mice. MNC secretome treated mice showed a trend towards decreased CD4+ cell counts and higher numbers of CD4+/7-AAD positive cells. This observation, however, did not reach significance (n=4-5).

Discussion

Myocarditis is one of the leading causes for dilated cardiomyopathy and therefore a major health issue worldwide. The pathophysiology underlying the disease is still not completely understood, however, autoimmunity is considered a key factor in the persistence of the myocardial inflammatory damage. According to this hypothesis the idea emerged that myocarditis can be cured by immunosuppression. A very early attempt in this direction were studies on glucocorticoid treatment in the late 80s (116). In a first randomized controlled trial, Parillo *et al.* evaluated 102 patients with dilative cardiomyopathy. 60 of the patients included in the study had an evidence of inflammation, 62 patients had no evidence of inflammation in endomyocardial biopsies. Both groups were randomized to receive either prednisolone or no prednisolone. Although a higher percentage of patients receiving prednisolone in the inflammatory group initially had better left ventricular ejection fractions, this effect evened out at the 6 and 9 months evaluations – most likely due to spontaneous restoration of cardiac function in the control group.

The Myocarditis Treatment Trial (MTT) was the next large randomized controlled trial addressing the question of immunosuppression in the treatment of myocarditis. A placebo group was compared to a group receiving prednisolone and azathioprine or prednisolone and cyclosporine (117). The mean change in left ventricular ejection fraction at 28 weeks did not differ between the group of patients on immunosuppressive therapy and the control group. These two studies suggest that immunosuppression should not be prescribed for the routine treatment of acute, viral myocarditis, and immunosuppressive regime is therefore not recommended in the guidelines (118, 119).

In contrast to this, immunosuppression may be beneficial during the chronic phase of myocarditis. Wojnicz *et al.* treated patients with chronic myocarditis, proven by increased human leukocyte antigen (HLA) expression on heart biopsy, with either prednisolone and azathioprine or placebo for three months. Left ventricular ejection fraction improved significantly in the immunosuppression group, even two years after treatment (120). This finding could be confirmed by Frustaci *et al.* (121, 122).

The development of myocarditis animal models led to further insights in the pathophysiology, diagnosis and treatment of the disease. Clinical observations in patients suffering from dilative cardiomyopathy evidenced increased levels of anti-viral antibodies (123). This finding was translated into animal models, injecting mice strains of cardiotropic virus – e.g. encephalomyocarditis virus or coxsackievirus B. Severe myocarditis was induced in susceptible mouse strains e.g. BALB/c, DBA/2, and C3H/He mice (124). However, the development of a myocardial infiltrate was not a stable finding and was influenced by many host factors including malnutrition, exercise, sex, sex hormones and age (81, 125, 126). The EAM model is based on a different assumption and concentrates on autoimmune aspects of myocarditis. It was first described by Pommerer *et al.* (101). In contrast to the “classical” way of myocarditis induction by cardiotropic viruses which leads to myocardial damage in only a minority of cases, the EAM method results in the development of myocarditis in nearly 100% of animals. CD4⁺ cells are considered pivotal in the EAM model, since depletion of CD4⁺ cells completely abrogates myocardial infiltration (103).

Since CD4⁺ cells play a key role in the EAM model we performed proliferation experiments of highly purified CD4⁺ cells treated with MNC secretome. The proliferative response towards anti-CD3 stimulation and in a mixed lymphocyte reaction was dose-dependently inhibited by adding MNC secretome to the experimental setting. The reason for this dampened proliferative response was a strong and stable induction of apoptosis via the external pathway of apoptosis.

Auto-antibody formation is a well described feature in the pathogenesis of myocarditis. Antibodies against a wide range of receptors, mitochondrial and contractile proteins have been found both in humans and in animal models (127). Myosin-specific antibodies are detectable in 46% of sera from patients suffering from a dilative cardiomyopathy in western blot analysis (128). In contrast to that, auto-antibody formation is not directly involved in the pathophysiology of myocardial infiltrates in the EAM model. B-cell deficient mice still develop a myocarditis (114). However, levels of anti-myosin

antibodies can be considered a surrogate marker to monitor disease severity in the EAM model. In our study decreased circulating anti-myosin IgG₁ and IgM together with reduced levels of IL-1 β and IL-6 underline the therapeutic effect of MNC secretome.

A therapeutic T cell depletion was shown to be an effective way to reduce myocardial damage in animal models. Rats treated with an anti-CD4 monoclonal antibody had significantly improved functional parameters in echocardiographical evaluations. In addition, lymphocytes obtained from treated animals showed no proliferative response after *in vitro* stimulation with a myosin fragment (104, 106). In a tedious work by Valaperti *et al.* the importance of CD4⁺ cell function was addressed by showing that injection of CD11b⁺ monocytes, suppressed the CD4⁺ dependent, MyHC α -specific autoimmune response (103). These findings are supported by the clinical observation that T cell depletion is a possible rescue therapy for fulminant autoimmune myocarditis (110, 111, 129).

To the best of our knowledge this is the first study evaluating immunosuppressive features of a high-dose application of MNC secretome in the murine EAM model. Further studies in large animal models are warranted to perpetuate the concept of using MNC secretome for the treatment of myocarditis and extrapolate our findings to other autoimmune diseases.

CONSIDERATIONS ON USING PBMC/MNC SECRETOME FOR THERAPEUTIC PURPOSES

Secretome research and the application of conditioned medium for the therapy of ischemic and inflammatory diseases is a fast growing field of regenerative medicine. Initially only secretomes derived from stem cells have been investigated, however, increasing evidence can be found in the literature that the beneficial effects of secretome therapy is not restricted to stem cells. We and others have shown that the secretome of stem cells only marginally differs from the secretome of other cell types (14, 15, 98). The major advantage of using PBMC/MNC instead of stem cells is that they can be obtained by a simple blood draw. Therefore PBMC/MNC secretome can be produced easily and in large quantities. The dialysis and lyophilization process was implemented in a standardized production process to be able to store the compound until needed. This makes an off-the shelf utilization possible, an important aspect for a later clinical application.

Using conditioned medium instead of cells for therapeutic purposes has another major advantage: as a cell-free treatment, the risk of adverse immunologic reactions are minimal, infectious risks and other potential long-term negative effects caused by the presence of exogenous cells can be minimized.

Nearly all of the experiments shown in this work were performed with secretome obtained in an allogeneic fashion from healthy individuals. The question whether MNC secretome obtained from e.g. patients during AMI have the same effect is currently addressed in our laboratory. If both, secretome from healthy and diseased individuals are similar effective, an autologous application would be the next logical step.

In the clinical scenario MNC secretome - as a “biological” - can be reconstituted in physiological saline solution and administered like a conventional intravenous infusion therapy. Comparable blood derived products e.g. intravenous immunoglobulin preparations or plasma derived coagulation factors have confirmed their clinical usefulness over the last decades. Before testing PBMC/MNC secretome in humans some important items have to be addressed. The complete production process has to comply with good manufacturing practice (GMP) guidelines. Viral inactivation steps have to be introduced and since secretomes are produced from different donors, tests evaluating the reproducibility of effects have to be included in a quality control protocol. This will hopefully lead off an approval of the medical authorities and pave the way for first phase I/II clinical trials.

CONFLICT OF INTEREST

The author of this thesis declares no conflict of interest. This study was funded by the Christian Doppler Research Association, APOSIENCE AG and the Medical University of Vienna. The Medical University of Vienna has claimed financial interest. H.J.A. is a shareholder of APOSIENCE AG, which owns the rights to commercialize PBMC and MNC secretome for therapeutic use.

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 ÖGAI 2009, Salzburg, Austria
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 Österreichischer Chirurgenkongress 2010, Linz, Austria
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 12th Central European Lung Cancer Conference 2010, Budapest, Hungary
 2nd EACTS Meeting on Cardiac and Pulmonary Regeneration 2010, Vienna, Austria
 19th European Conference on General Thoracic Surgery (ESTS) 2011, Marseille, France
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 Synthes Wet Lab. New Technologies in Sternal and Chest Wall Stabilization, 2011, Stuttgart, Germany
 Operative Therapie des laryngotrachealen Übergangs, 2012, Stuttgart, Germany
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 Observer: University of Toronto, Division of Thoracic Surgery, 07/2012
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 Travel award – 3rd TERMIS World Congress 2012

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Presentations (35):

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Posterausstellung anlässlich der vfwf-Universitätsvorlesung "Die Medizinische Universität der Zukunft" 2006, Wien. Caspase-cleaved cytokeratin 18 and 20S proteasome in liver degeneration (poster presentation)

Posterausstellung anlässlich der vfwf-Universitätsvorlesung "Die Medizinische Universität der Zukunft" 2006, Wien. Case report: rabbit antithymocyte globulin (rATG) and ECMO bridge as new options in the treatment of giant cell myocarditis (poster presentation)

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Austrotransplant 2006, Hof. Cytotect (CMV hyperimmunoglobuline) – passive immunisation, active immune suppression (oral presentation)

Kardiovaskuläre Forschungstage 2007, Weissensee. Elevated levels of interleukin-1 β -converting enzyme and caspase-cleaved cytokeratin-18 (ccCK-18) in acute myocardial infarction (oral presentation)

International Society for Heart and Lung Transplantation 27th Annual Meeting 2007, San Francisco. Anti-proliferative properties of CMV hyperimmunoglobulin are related to activation induced cell death *in vitro*: Possible role in tolerance induction (poster presentation, mini oral presentation)

Morning lectures of the Dept of Cardiothoracic Surgery, Medical University of Vienna 2007. CMV hyperimmunoglobulin - Role In Allo-Immune Response (oral presentation)

Österreichischer Chirurgenkongress 2007, Graz. Gigantic coronary fistula: rare finding without clinical symptom (poster presentation)

Österreichischer Chirurgenkongress 2007, Graz. Heat shock proteins 27/60/70/90 α and 20S proteasome in on- versus off-pump coronary artery bypass graft patients (oral presentation)

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Austrotransplant 2007, St. Wolfgang. CMV hyperimmunoglobulin influence NK cell viability and function *in vitro* (poster presentation)

Austrotransplant 2007, St. Wolfgang. CMV hyperimmunoglobulin role in allo-immune response (invited lecture)

EAACI-GAZLEN Davos Meeting 2008, Pichl. CMV Ig and IVIg Induce CD32-Mediated Platelet Aggregation *in vitro*: Implication of Therapy Induced Thrombocytopenia and Thrombosis *in vivo* (poster presentation)

Österreichischer Chirurgenkongress 2009, Wien. ASD Repair after a 10-month Treatment with Bosentan in a Patient with severe pulmonary arterial Hypertension (poster presentation)

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Österreichischer Chirurgenkongress 2010, Linz. Considerations on infectious complications using a drowned lung for transplantation (poster presentation)

Österreichischer Chirurgenkongress 2010, Linz. Seldom referral to the thoracic surgeon: spontaneously ruptured left inferior thyroid artery (poster presentation)

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Österreichischer Chirurgenkongress 2010, Linz. Extended fore quarter amputation and chest wall resection for treatment of malignant tumors around the shoulder: A single-center experience (oral presentation)

12th Central European Lung Cancer Conference 2010, Budapest. Curative Resections of Pancoast Tumors – where do we stand? (oral presentation)

Österreichischer Chirurgenkongress 2011, Vienna. Curative Resections of Pancoast Tumors (oral presentation)

International Society for Heart and Lung Transplantation 32nd Annual Meeting 2007. Prague. Chronic inflammatory changes and no evidence of chromosomal aberrations in Mounier-Kuhn syndrome: report of a case (poster presentation)

International Society for Heart and Lung Transplantation 32nd Annual Meeting 2007. Prague. Secretome of apoptotic peripheral blood cells attenuates microvascular obstruction in acute myocardial infarction: role of platelet aggregation (oral presentation)

9. PneumoUpdate 2012, Innsbruck. Kurative Resektionen von Pancoast-Tumoren (poster presentation)

Österreichischer Chirurgenkongress 2012, Salzburg. Secretome of apoptotic peripheral blood cells (APOSEC) attenuates microvascular obstruction in a porcine closed chest reperfused acute myocardial infarction model: role of platelet aggregation in vitro and in vivo (oral presentation)

Österreichischer Chirurgenkongress 2012, Salzburg. Secretome of apoptotic peripheral blood cells (APOSEC) induces coronary vasodilation: impact on microvascular obstruction during acute myocardial infarction (oral presentation)

Österreichischer Chirurgenkongress 2012, Salzburg. Secretome from mononuclear cells confers immunosuppression in a murine autoimmune myocarditis model (oral presentation)

Österreichischer Chirurgenkongress 2012, Salzburg. Impact of pulmonary metastasectomy on lung function parameters (oral presentation)

ESC Congress 2012, Munich. Secretome of mononuclear cells confers immunosuppression in a murine autoimmune myocarditis model (poster presentation)

ESC Congress 2012, Munich. Secretome of apoptotic peripheral blood cells (APOSEC) attenuates microvascular obstruction in a porcine closed chest reperfused acute myocardial infarction model: role of platelet aggregation and vasodilation (oral presentation)

3rd TERMIS World Congress 2012, Vienna. Secretome of apoptotic peripheral blood cells (APOSEC) attenuates microvascular obstruction in a porcine closed chest reperfused acute myocardial infarction model: role of platelet aggregation and vasodilation (poster presentation)

3rd TERMIS World Congress 2012, Vienna. Secretome from mononuclear cells confers immunosuppression in a murine autoimmune myocarditis model (poster presentation)

Studies approved by the institutional review board of the Clinical Research Ethics Committee of the Medical University of Vienna (17):

Search for apoptosis specific activation marker and TH2 cytokine profile as surrogate markers for bronchiolitis Obliterans (BO) syndrome: implications for treatment and early detection of BO in lung transplant recipients

Roth G, Klepetko W, Pollreisz A, Hoetzenecker K, Brunner M, Ankersmit HJ – 2004

Investigation of T-Cell Response to Industrial Glutaraldehyde Fixed Porcine Valves: The Role of Specific Immune System in Degeneration During Adult Aortic Valve Replacement Therapy

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Lambers C, Hacker S, Hoetzenecker K, Pollreisz A, Ankersmit HJ – 2006

In Vitro Investigation of Immunosuppressive Effects of CVM hyperimmunoglobulin

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Jaksch P, Klepetko W, Pollreisz A, Hacker S, Hoetzenecker K, Ankersmit HJ – 2006

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Moser B, Nickl S, Hacker S, Hoetzenecker K, Lichtenauer M, Lamber C, Arns-Dietl BM, Klepetko W, Ankersmit HJ – 2008

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Studies approved by the Animal Experiment Committee according to the animal experiment ordinance of the Austrian Federal Ministry for Education, Science and Culture (5):

Immunmodulation nach Herzinfarkt, untersucht an der Ratte

Podesser B, Ankersmit HJ, Hoetzenecker K, Losert U – 2006

Immunmodulation auf Zell- und Zytokinebene nach Herzinfarkt, untersucht an der Ratte

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Scheed A, Hoetzenecker K, Aigner C, Klepetko W – 2011

National Co-operations:

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Academic Teaching:

VO AHA Guidelines in der Cardiovasculären Chirurgie 2007 – invited lecture

VO+SE Vom Textbuch der Herzchirurgie zur PowerPoint-Präsentation 2007 – invited lecture

MedSuccess 2008: IVIG und Thrombozytenaggregation – Hyperviskosität als Ausrede?

MedSuccess 2009: CMV Prophylaxe in der Transplantation

MedSuccess 2009: COPD - eine Autoimmunerkrankung?

MedSuccess 2010: Über Studium, Forschung und akademische Karriere

MedSuccess 2010: COPD - eine Autoimmunerkrankung?

KP: Tertial 4 - Chirurgische Fächer WS 2011/2012

SE Methoden der chirurgisch-immunologischen Forschung WS 2011/2012

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Clinical Investigations:

Multi-center, double-blind, randomized, placebo-controlled, parallel-group study to assess the efficacy, safety and tolerability of tezosentan in patients with pre-operative pulmonary hypertension, due to left heart disease, undergoing cardiac surgery

Study site coordinator of the Dept of Cardiothoracic Surgery, MUV

Sponsor: Actelion

2007-2008

Normothermic ex vivo lung perfusion – improved evaluation of donor lungs prior to transplantation and impact on primary graft dysfunction

Co-investigator

Sponsor: –

2010-2011

Review activity:

American Journal of Transplantation since 2007

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ORIGINAL CONTRIBUTION

Secretome of apoptotic peripheral blood cells (APOSEC) attenuates microvascular obstruction in a porcine closed chest reperfused acute myocardial infarction model: role of platelet aggregation and vasodilation

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Abstract Although epicardial blood flow can be restored by an early intervention in most cases, a lack of adequate reperfusion at the microvascular level is often a limiting prognostic factor of acute myocardial infarction (AMI). Our group has recently found that paracrine factors secreted from apoptotic peripheral blood mononuclear cells (APOSEC) attenuate the extent of myocardial injury. The aim of this study was to determine the influence of APOSEC on microvascular obstruction (MVO) in a porcine AMI model. A single dose of APOSEC was intravenously injected in a closed chest reperfused infarction model. MVO was determined by magnetic resonance imaging and cardiac catheterization. Role of platelet function and

vasodilation were monitored by means of ELISA, flow cytometry, aggregometry, western blot and myographic experiments *in vitro* and *in vivo*. Treatment of AMI with APOSEC resulted in a significant reduction of MVO. Platelet activation markers were reduced in plasma samples obtained during AMI, suggesting an anti-aggregatory capacity of APOSEC. This finding was confirmed by *in vitro* tests showing that activation and aggregation of both porcine and human platelets were significantly impaired by co-incubation with APOSEC, paralleled by vasodilator-stimulated phosphoprotein (VASP)-mediated inhibition of platelets. In addition, APOSEC evidenced a significant vasodilatory capacity on coronary arteries via p-eNOS and iNOS activation. Our data give first evidence that APOSEC reduces the extent of MVO during AMI, and suggest that modulation of platelet activation and vasodilation in the initial phase after myocardial infarction contributes to the improved long-term outcome in APOSEC treated animals.

K. Hoetzenecker and A. Assinger contributed equally to this work. I. Volf and H.J. Ankersmit contributed equally to this work.

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Introduction

Myocardial infarction remains one of the major health issues worldwide. Early reperfusion of the culprit coronary artery within a narrow time window by percutaneous coronary intervention (PCI) and fibrinolytic agents has significantly improved early mortality [59]. Although tremendous efforts have been made in replacing infarcted myocardium, so far no therapy has proven effective in clinical application. The induction of myocardial repair by progenitor cells was suggested a promising strategy based on encouraging data from animal models [15, 23, 47, 48, 63]. However, the efficacy of stem cells as therapeutic agents in human AMI is currently under scrutiny [22, 26, 40, 62]. Based on recent observations showing that the infusion of cultured apoptotic peripheral blood mononuclear cells (PBMC) was able to prevent experimental AMI in rodents [4, 38] we speculated whether paracrine factors secreted from PBMC—termed APOSEC (abbreviation for APOptotic cell SEcRetoma)—are capable to attenuate AMI in a rodent and in a closed chest porcine ischemia/reperfusion AMI model. By a single intravenous infusion of APOSEC, scar tissue formation was significantly reduced. Additionally, an improvement of haemodynamics with higher values of ejection, and a better cardiac output was found in magnetic resonance imaging (MRI) analyses. A possible mode of action was suggested by showing that co-incubation of primary human cardiomyocytes with APOSEC led to an activation of pro-survival signalling-cascades (AKT, Erk1/2, CREB, c-Jun), and increased anti-apoptotic gene products (Bcl-2, BAG-1) in vitro, consequently protecting cardiomyocytes from starvation-induced cell death [39].

However, this ascribed mechanism only partially explains the beneficial effects of the “biological” APOSEC in AMI. Although coronary blood flow is re-established after PCI, no-reflow phenomena impair the beneficial effect of reperfusion due to microvascular obstruction (MVO) [51]. The two major pathophysiological mechanisms associated with MVO are enhanced platelet activation in the microcirculation and coronary vasoconstriction. Clinical reports have evidenced that platelet activation is directly correlated with the severity of myocardial damage after AMI [8, 16, 17]. Besides, there is sufficient evidence that the vasomotor state in the coronary vasculature is closely linked to the no-reflow phenomenon [50, 60]. Based on these accepted pathophysiological concepts we speculated whether APOSEC treatment has an effect on the development of hypoxia-induced MVO.

Here, we provide evidence that intravenous application of APOSEC attenuates myocardial infarction by reducing MVO in a porcine closed chest ischemia/reperfusion AMI model. Moreover, we show that APOSEC is an anti-aggregatory compound and has vasodilatory properties.

Materials and methods

Generation of porcine and human APOSEC

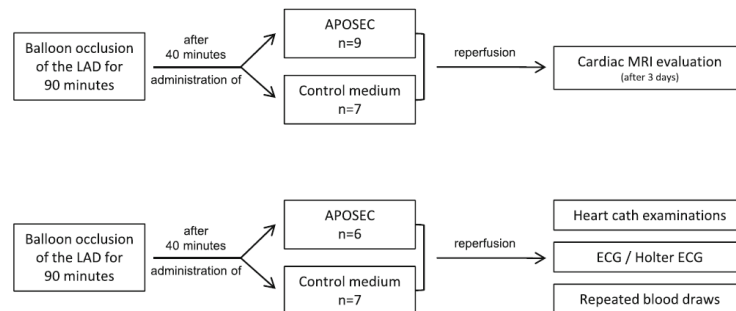
For large animal experiments, blood was obtained from pigs by direct heart puncture under sterile conditions. Peripheral blood mononuclear cells were purified by Ficoll-Paque (GE Healthcare Bio-Sciences AB, Sweden) density gradient centrifugation. Apoptosis of PBMC was induced by Caesium-137 irradiation with 60 Gray (Gy) and PBMC were resuspended in CellGro serum-free medium (Cell Genix, Freiburg, Germany; 25×10^6 cells/ml). After incubation for 24 h supernatants were dialyzed against ammonium acetate (at a concentration of 50 mM), sterile filtered, frozen and lyophilized. APOSEC from four different donor pigs were pooled for further experiments.

For in vitro experiments PBMC obtained from young healthy volunteers (APOSEC healthy), patients suffering from insulin-dependent diabetes (APOSEC DM), or patients with congestive heart failure NYHA>III (APOSEC CHF) were used (ethics committee vote: EK-Nr 2010/034; 2009/352). Secretome was produced according to the protocol described above; cells were cultured at a concentration of 1×10^6 cells/ml for platelet and a concentration of 2.5×10^6 cells/ml for HUVEC experiments. UltraCulture (Cambrex Corp., North Brunswick, NJ, USA) served as the carrier medium. APOSEC pooled from six to seven donors was used for the respective experiments.

Porcine closed chest reperfused infarction model and administration of APOSEC

Animal experiments were approved by the University of Kaposvar, Hungary (vote: 246/002/SOM2006, MAB-28-2005). Two experimental settings were designed (Fig. 1). Pigs (female large whites weighing approximately 30 kg) received 75 mg clopidogrel and 100 mg acetylsalicylic acid as a premedication. At the day of intervention animals were sedated with 12 mg/kg ketamine hydrochloride, 1.0 mg/kg xylazine and 0.04 mg/kg atropine. A Maverick balloon catheter (diameter: 3.0 mm, length: 15 mm; Boston Scientific, Natick, USA) was inserted into the left anterior descending artery (LAD) and inflated after the origin of the second major diagonal branch; ST segment abnormalities were recorded by electrocardiography

Fig. 1 Flow charts of the two experimental settings of the porcine acute myocardial infarction experiments



(ECG). ST-segment resolution was calculated as an ST-segment decrease of $>50\%$ of the initial ST-segment elevation. Additionally, pigs were monitored by Holter ECG during ischemia and until 60 min after reperfusion (Gepa-Med, Vienna, Austria). Forty minutes after the start of the LAD occlusion, the lyophilized secretome from 1×10^9 irradiated apoptotic porcine PBMC or lyophilized serum-free cell culture (resuspended in 250 ml of 0.9 % NaCl solution) was administered intravenously over 25 min. After 90 min occlusion, the balloon was deflated and reperfusion was established. Control coronary angiography was performed to prove the patency of the infarct-related artery and to exclude arterial injury. Euthanasia was performed by the administration of saturated potassium chloride 24 h or 3 days after AMI induction.

Magnetic resonance imaging

MRI imaging was performed on day three with a 1.5-T clinical scanner (Avanto, Siemens, Erlangen, Germany). Planimetric analysis of MRI images was performed using QMass software (Medis, Leiden, The Netherlands). Similarly to other studies [2], the presence of MVO was evaluated by observing the late hypo-enhancement within a hyper-enhanced region on late enhancement MRI images, 10 min after the administration of intravenous Gadolinium based contrast agent. Previously, infarcted areas were semi-automatically segmented by thresholding the left ventricular myocardium to the mean $+2 \times$ SD values of unaffected myocardium. MVO was manually assessed for each subject when areas within the infarcted areas presented low signal intensity (i.e., “dark zones” within “bright” zones). Manual planimetry was used to define the area of MVO for each slice and then areas were multiplied by the slice thickness (8 mm) to get volumetric measurements. Results are given in volume values (cm^3).

Bari score analysis

To verify comparable basic conditions between groups prior to balloon occlusion, Bari scores were calculated for all animals based on LAD and CX pre-occlusion angiogram according to the method previously described [49].

APOSEC content evaluation

APOSEC produced from healthy donors, diabetic patients and CHF patients was evaluated for levels of IL-8, ENA-78, VEGF (all DuoSet kits, R&D systems, Minneapolis, USA) following the manufacturer’s instructions. Nitric oxide (NO) was determined by measuring decomposition products nitrite and nitrate with a commercially available colorimetric assay kit (Abcam, Cambridge, UK).

In vivo platelet function during ischemia/reperfusion

Plasma samples (3.8 % trisodium citrate tubes) were obtained by a venous draw before occlusion (0 h), before balloon deflation (90 min), after reperfusion (240 min) and after 24 h. Secreted platelet activation markers sCD40L, sCD62P, platelet factor-4 (PF-4) and thrombospondin-1 (TSP-1) were measured using commercially available ELISA kits (Uscn, Wuhan, China).

In vitro platelet function analyses

Human platelet isolation

Blood was drawn from eight healthy human volunteers, who declared to be free of any medication for at least 2 weeks. All blood donors gave their informed written consent to the study. They were venipunctured with a 20-G needle and the blood was anticoagulated with one/ten volume of 3.8 % (w/v) trisodium citrate. Immediately after

collection, blood was centrifuged at 125 g for 20 min to obtain platelet rich plasma (PRP). To avoid contamination with other cell types only the upper two-thirds of the PRP fraction were used. Platelets were purified by gel filtration using Sepharose 4B columns with HEPES-Tyrode buffer containing 0.5 % human serum albumin as previously described [6]. Experiments with porcine platelets were performed with platelet-rich plasma.

Measurement of platelet activation

Isolated platelets were pre-incubated with APOSEC of 2×10^5 cultured cells for 10 min and then stimulated for 5 min with thrombin receptor-activating peptide TRAP-6 (BACHEM, Basel, Switzerland), adenosine diphosphate (ADP; Sigma-Aldrich Corp., St Louis, MO, USA) or collagen (MoeLab, Langenfeld, Germany). Platelets were then either incubated with PE labeled anti-CD62P antibody, FITC-labeled anti-CD63 or FITC-labeled anti-CD40L (Becton–Dickinson, Austria) for 30 min, followed by fixation in 1 % formaldehyde and then analyzed by flow cytometry (FACSCalibur, Becton–Dickinson, Austria).

Platelet aggregation experiments

Platelet-rich plasma was stirred in the presence or absence of APOSEC from 2×10^5 cultured cells in an optical 4-channel aggregometer (490-4D, Chronolog Corp., Havertown, PA, USA) at 37 °C for 5 min, thereafter the indicated agonists were added and changes in light transmission recorded over 10 min. After this period, theophylline (300 μ M) and adenosine (500 μ M) were added to stop further activation. Platelets were centrifuged at 1,000 g for 2 min to obtain supernatant which was analyzed for soluble CD62P, soluble CD40L and thrombospondin (TSP-1) content. ELISA tests for sP-selectin (Quantikine; R&D Systems, Minneapolis, MN, USA) and sCD40L (Bender MedSystems, Vienna, Austria) were performed according to manufacturers' instructions. Thrombospondin-1 was determined by immunoblotting, as previously described [58].

Quantification of intraplatelet VASP phosphorylation

Isolated platelets were incubated with different concentrations of prostaglandin E₁ (PGE₁) and APOSEC (2×10^5) for 2 min followed (if indicated) by 5 min of incubation with ADP. Cells were fixed in 1 % formaldehyde for 10 min, permeabilized with 0.5 % triton X-100 and incubated for 45 min with monoclonal antiphospho VASP antibody, clone 22E11 (nanoTools, Teningen, Germany), which detects VASP phosphorylation at serine 239. After a washing step, platelets were incubated with

secondary fluorescein isothiocyanate (FITC) conjugated polyclonal anti-mouse IgG antibody (Becton–Dickinson) for 30 min and analyzed by flow cytometry.

In vivo measurements of vasodilatory mediators during ischemia/reperfusion

Plasma samples (3.8 % trisodium citrate tubes) obtained before LAD occlusion, 90 min after occlusion, after reperfusion and 24 h after AMI induction were evaluated for different vasodilatory mediators. Systemic levels of prostacyclin (PGI₂) and vasoactive intestinal peptide (VIP) were determined by ELISA technique (Uscn, Wuhan, China; antibodies-online, Aachen, Germany). Nitric oxide was determined as described above.

In vitro analyses of vasodilatory effects of APOSEC

HUVEC culture and immunoblot analysis

Primary human umbilical vein endothelial cells (HUVEC) were obtained from CellSystems (CellSystems Biotechnologie, Troisdorf, Germany) and cultured in endothelial cell growth medium (EGM-2, Lonza, Basel, Switzerland) at 37 °C. For Western Blot analysis, 3×10^5 cells were seeded in six-well plates and cultured in EGM-2 medium. 24 h later, cells were incubated with lyophilized APOSEC from 2.5×10^6 cells or lyophilized control medium, resolved in EGM-2 medium without growth factors, for 60 min (phospho-eNOS detection) or 24 h (iNOS detection). Western blot analysis was performed as described previously [45]. Briefly, HUVEC were lysed in SDS-PAGE loading buffer, sonicated, centrifuged, and denatured before loading. SDS-PAGE was conducted on 8–18 % gradient gels (GE Healthcare, Uppsala, Sweden). The proteins were then electro-transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). Immunodetection was performed with either a rabbit polyclonal anti-inducible nitric oxide synthase (iNOS) antibody (Cell Signaling Technology, Inc., Danvers, MA, USA), phospho e-NOS antibody (Cell Signaling Technology, Inc.) or a mouse monoclonal anti-GAPDH antibody (Acris, Herford, Germany) followed by horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse IgG antisera (both 1:10,000; GE Healthcare). Reaction products were detected by chemiluminescence with the ChemiGlow reagent (Biozyme Laboratories Limited, South Wales, UK) according to the manufacturer's instructions.

Coronary perfusion assay

Coronary perfusion assay was performed as described previously [1]. Hearts were obtained from untreated,

sacrificed domestic pigs and transferred to the laboratory in a modified Krebs-Henseleit buffer solution. Coronary arteries were dissected from the heart and cut in 4 mm thick rings. Each coronary segment was mounted in a temperature-controlled 10 mL tissue bath containing a modified Krebs-Henseleit buffer solution. To measure circular wall tension, the rings were suspended between two L-shaped pins in a myograph. After approximately 1 hour, vessels were contracted with endothelin-1 (30 nM; Calbiochem, Darmstadt, Germany). APOSEC was added to the probes in different concentrations (dose escalation) and changes in arterial wall tension were measured. In some experiments NOS inhibitor L-NG-Nitro arginine methyl ester (L-NAME) was added.

Immunohistochemical evaluation of coronary rings

Coronary rings isolated according to the above described procedure were incubated for 60 min in the presence or absence of APOSEC. Rings were fixed in 10 % neutrally buffered formaldehyde solution and embedded in paraffin. The tissue samples were stained with hematoxylin-eosin (HE). For immunohistochemical stainings an antibody recognizing eNOS, phosphorylated at Ser 1,177 (Biorbyt, Cambridge, UK) was applied. Tissue samples were evaluated on an Olympus AX70 microscope (Olympus Optical Co. Ltd., Tokyo, Japan) and captured digitally using Meta Morph v4.5 software (Molecular Devices, Sunnyvale, USA).

Statistical analysis

Results are depicted as mean \pm standard error of the mean and were analyzed by student's *t* test or repeated measures analysis of variance (ANOVA) followed by Bonferroni correction. Data analysis was performed with SPSS 18.0 (SPSS inc., United States). A *p* value less than 0.05 was regarded as statistically significant (asterisk indicates $p < 0.05$; double asterisk indicates $p < 0.01$).

Results

APOSEC reduces MVO in a porcine AMI model

APOSEC has recently been shown to effectively reduce myocardial damage during AMI [39]. To define the impact of APOSEC on MVO, pigs were evaluated 3 days after myocardial infarction by MRI. Areas of MVO were significantly lower in pigs treated with APOSEC when compared to control animals (Table 1; APOSEC: $0.3 \pm 0.1 \text{ cm}^3$; control: $0.8 \pm 0.1 \text{ cm}^3$; $p = 0.04$). This finding was confirmed by cardiac catheterization, as the

Table 1 MVO analysis

	Group	MVO (cm^3)	Qualitative
1	APOSEC	0	Not visible
2	APOSEC	0.427	Small
3	APOSEC	0	Not visible
4	APOSEC	1.24	Small
5	APOSEC	0	Not visible
6	APOSEC	0.56	Small
7	APOSEC	0	Not visible
8	APOSEC	0	Not visible
9	APOSEC	0.91	Small
10	Control	0.86	Small
11	Control	0.76	Small
12	Control	1.04	Small
13	Control	0.26	Very small
14	Control	0.96	Small
15	Control	0.72	Small
16	Control	0.97	Small

Pigs were evaluated 3 days after induction of AMI for areas of MVO. APOSEC treated animals had significant smaller areas of impaired microvascular perfusion when compared to control animals (APOSEC: 0.3 ± 0.1 ; control: 0.8 ± 0.1 ; $p = 0.04$)

Table 2 Cardiac catheterization analysis

	Control	APOSEC	<i>p</i> value
Corr. TMI frame count	44.4 ± 3.6	28.7 ± 1.9	0.022
Myocardial blush grade	1.3 ± 0.3	2.5 ± 0.3	0.033

Corrected TIMI frame counts were lower in animals treated with APOSEC indicating a good microvasculature perfusion ($p = 0.022$). Additionally, animals from the APOSEC group had a significantly higher myocardial blush grade than control pigs ($p = 0.033$)

$n = 6-7$

corrected thrombolysis in myocardial infarction (TIMI) frame count was significantly lower in APOSEC treated animals (28.7 ± 1.9 vs. 44.4 ± 3.6 ; Table 2). In addition, the myocardial blush grade, which directly reflects myocardial tissue perfusion, was significantly better in APOSEC treated pigs (mean grade 1.3 ± 0.3 vs. 2.5 ± 0.3 ; Table 2).

BARI scores

To rule out the possibility that differences in MVO could be a result of differences in the coronary vascularisation, Bari scores were determined from pre-interventional angiographies. A homogenous distribution of coronary vessels was found between groups (Suppl. Fig. 1a).

Area at risk measured by MRI

During the early phase of AMI, the area at risk (AAR) determines the zone of ischemic injured myocardium. To confirm that ischemic areas were comparable in both groups we analyzed T2-weighted images in the MRI analysis 3 days after LAD occlusion. No differences in the AAR could be found between the two groups (control: 22.9 ± 2.2 vs. APOSEC: 20.2 ± 1.4 ; $p = 0.294$; Suppl. Fig. 1b), evidencing that the size of hypoperfused myocardium at the time of the ischemic episode was similar in the groups.

Haemodynamic monitoring and ECG data

Haemodynamic monitoring showed a trend towards better left ventricle contraction capacity (dP/dt/P) in APOSEC group, as compared to control group (27.2 ± 20.6 vs. $17.4 \pm 4.0 \text{ min}^{-1}$). On-line ECG monitoring during coronary occlusion and reperfusion showed ST segment resolution in four out of six animals in the APOSEC group compared to only one out of seven pigs in the medium group. Holter ECG evaluations revealed a reduction of ventricular arrhythmias (expressed in total number of extrasystoles, couplet, triplet, and ventricular tachycardias) during coronary occlusion and the perfusion period (Table 3).

APOSEC inhibits platelet aggregation in vivo and in vitro

Since platelets are the major contributor to MVO we hypothesized that APOSEC has a direct influence on platelet function. Systemic platelet activation markers in plasma, obtained at different time points after AMI induction, were measured. Levels of sP-selectin, TSP-1, PF-4 and sCD40L were lower in the APOSEC group when compared to control animals (Fig. 2a–d). These in vivo findings were confirmed by in vitro experiments.

Isolated porcine and human platelets were stimulated with different concentrations of collagen, ADP and TRAP-6 with or without pre-incubation of APOSEC. As measured by light transmittance aggregometry, platelet aggregation could be inhibited by the addition of APOSEC (Fig. 3a, b), both in a maximal and a half-maximal stimulation model.

The inhibitory effect of APOSEC on platelets was further characterized by measuring surface expression of different platelet activation markers. Levels of CD62P, CD63 and CD40L were significantly decreased after treating platelets with APOSEC, indicating an inhibitory role of APOSEC during platelet activation (Fig. 3c). These findings were corroborated by the evaluation of secreted activation factors in the supernatant of aggregation experiments. As determined by ELISA, concentrations of sCD40L and sCD62P were significantly lower after treating platelets with APOSEC (Fig. 3d). Thrombospondin has recently been described as a sensitive and stable parameter to monitor in vitro platelet activation. We therefore evaluated supernatants for amounts of secreted TSP-1 isoforms by western blots. There was a strong band of 140 kD TSP-1 detectable after ADP and TRAP-6 activation, which was reduced upon coincubation of platelets with APOSEC (Fig. 3e).

Enhanced VASP phosphorylation by APOSEC

VASP in its phosphorylated form represents a negative regulator of platelet activation. We could show that incubation of isolated human platelets with APOSEC led to an increase of intraplatelet phosphorylated VASP. In addition, coincubation of platelets with both APOSEC and different submaximal effective concentrations of PGE₁ increased VASP-phosphorylation in a synergistic way (Fig. 4). This is of special interest as PGE₁ represents a physiological relevant inhibitor of platelet function that acts through an increase of intraplatelet cAMP.

Table 3 Rhythmological evaluation

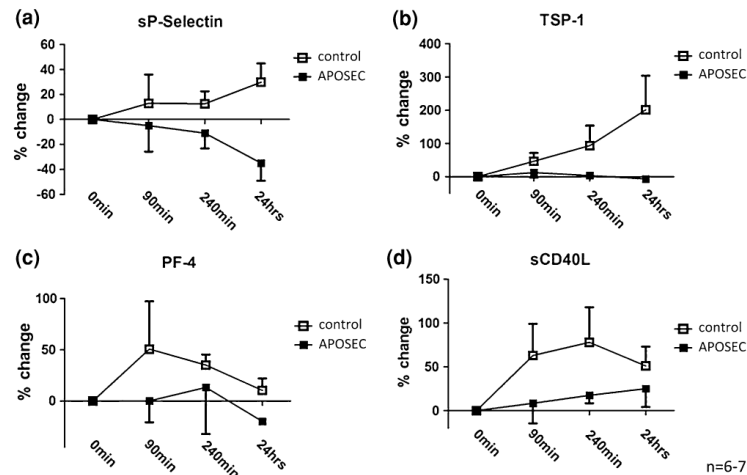
	ST-resolution		VES		Couplet		Triplet		VT	
	Control	APOSEC	Control	APOSEC	Control	APOSEC	Control	APOSEC	Control	APOSEC
During occlusion	–	–	238.7 ± 161.5	28.0 ± 11.0	10.7 ± 7.1	4.6 ± 3.0	10.7 ± 8.5	0.2 ± 0.2	5.7 ± 3.4	2.4 ± 1.9
After reperfusion	1/7	4/6	92.3 ± 31.0	49.0 ± 35.8	18.8 ± 8.6	8.0 ± 5.6	4.8 ± 3.3	3.4 ± 2.0	3.2 ± 1.9	3.4 ± 2.4

ECG and Holter-ECG analyses revealed lower rates of persisting ST abnormalities and significantly fewer episodes of arrhythmias in pigs receiving APOSEC

This was shown for VES ventricular extrasystole, couplets, triplets, VT ventricular tachycardia

$n = 6-7$

Fig. 2 Platelet activation markers. Soluble activation markers (sP-selectin, TSP-1, PF-4 and sCD40L) were reduced in APOSEC treated animals when compared to control pigs (a–d)



APOSEC induces coronary vasodilation

The role of vasodilators in the prevention and treatment of MVO is well described [33]. We therefore assessed vasodilatory mediators in serum samples obtained from the AMI animal model. Both, NO and PGI₂ were found to be increased after administration of APOSEC when compared to control animals (Fig. 5a). In addition, HUVEC upregulated iNOS expression after 24 h of co-incubation with APOSEC as determined by western blot. eNOS expression was not altered (data not shown), however, the active phosphorylated form of eNOS was increased 60 min after treating HUVEC with the compound (Fig. 5b). p-eNOS expression was also found elevated in coronary rings 60 min after treatment with APOSEC as determined by immunohistochemical stainings (Suppl. Fig. 4).

Finally, we evaluated direct vasodilatory effects of APOSEC. In APOSEC preparations NO was found in significant concentrations (12×10^6 mL: 39.5 nM; 1.2×10^6 mL: 16.9 nM; 0.12×10^6 mL: 1.3 nM), however, PGI₂ or VIP was not detectable. Myographic evaluations using isolated coronary arterial segments corroborated this finding. Treating coronary rings with APOSEC resulted in a significant dilation of the vessels in a dose dependent manner (Fig. 5c). This effect was not related to a de novo production of NO, since blocking NO synthesis with L-NAME had no effect on vasotonus (Suppl. Fig. 2).

APOSEC from healthy donors, diabetic patients and CHF patients show comparable properties

To address the question if the observed effects of APOSEC are limited to healthy donors, we produced APOSEC from

diabetic patients and patients suffering from CHF. Levels of three reference cytokines (IL-8, ENA-78, VEGF), which are known to be highly abundant in APOSEC [38], and NO were determined. No differences between APOSEC (healthy), APOSEC (DM) and APOSEC (CHF) were observed (Suppl. Fig. 3a). The functional relevance of this finding was further evaluated in platelet aggregation and vasodilation experiments. APOSEC (DM) and APOSEC (CHF) were similar effective in inhibiting platelet function (Suppl. Fig. 3b) and in inducing p-eNOS and iNOS expression in HUVEC (Fig. 5b) when compared to APOSEC (healthy).

Discussion

This study gives first evidence that APOSEC effectively reduces MVO in a clinically relevant ischemia/reperfusion AMI model. This finding was associated with an improvement in the myocardial blush grade and corrected TIMI frame count, two clinically established parameters of microvascular patency. Moreover, resolution of ECG alterations during experimental occlusion and reperfusion were mediated by treating animals with APOSEC. The impact of APOSEC on two major contributors of MVO was tested in vitro. Co-incubation of platelets and APOSEC led to an increase of phosphorylated VASP, consecutively inhibiting platelet aggregation in vitro. Treating HUVEC with APOSEC resulted in an induction of iNOS and p-eNOS. Additionally, direct vasodilatory effects of APOSEC were shown in myographical evaluations of isolated coronary arterial rings.

For a long time, beneficial effects in stem cell therapy were contributed solely to cellular mediated mechanism. Recently, this concept was challenged by works showing that paracrine signalling may be a significant additional mode of action [19, 41, 52, 53]. The importance of releasing pro-angiogenic and cytoprotective factors during AMI has already been shown for mesenchymal as well as for bone marrow derived stem cells [3, 14, 36]. We have recently expanded the concept of regenerative, paracrine factors derived from stem cell, by showing that the secretome of apoptotic PBMC attenuates myocardial infarction [39]. The major advantage of PBMC over stem cells is that they are a lot easier to access. Although secretome of stem cells and PBMC both mediate similar effects, their secreted factors slightly differ. In a protein chip array study Wollert and colleagues showed that out of 174 secreted factors, 25 factors were present in higher concentrations in bone marrow supernatants, and ten factors were found in higher concentrations in peripheral blood leucocytes [36]. To the best of our knowledge, our group was first to utilize the potential of paracrine factors derived from PBMC in an experimental AMI setting. Consequently, we have addressed features of APOSEC relevant for microvascular obstruction in this subsequent study.

After re-establishing blood flow in the occluded epicardial vessel, the integrity of the microcirculation in the vicinity of the post-ischemic myocardium is pivotal for a patient's prognosis. An open microvasculature was shown to supply infarct related myocardium with blood and avoiding myocyte necrosis [10, 42]. It is of utmost importance to maintain this residual blood flow within the AAR, since there is sufficient in vitro and in vivo evidence of viable myocardium hours to days after coronary occlusion [32, 44, 63]. If the preservation of microvascular flow fails, viable myocardium is gradually lost. It is a currently accepted notion that platelets are causative for microvascular dysfunction by releasing vasoconstrictive substances [21], by forming microemboli [28, 57] or by intravascular thrombus formation in the microcirculation [7]. Moreover, experimental evidence indicates that the detrimental effect of platelets is dependent on their activation status [64]. Relevant to MVO are the observations of Barrabes et al. [8], who showed in a porcine AMI model that ischemic injury triggers macro- and microvascular platelet deposition even in distant areas not related to the occluded coronary artery. This leads to impairment in coronary flow reserve and contractile function. With advances in understanding the pathophysiology of microvascular malperfusion, different therapeutic strategies inhibiting platelet function have been proposed. However, to date only the application of monoclonal antibodies blocking GPIIb-IIIa receptor improved microvascular flow and subsequently reduced infarct size in animal models [37]. This effect

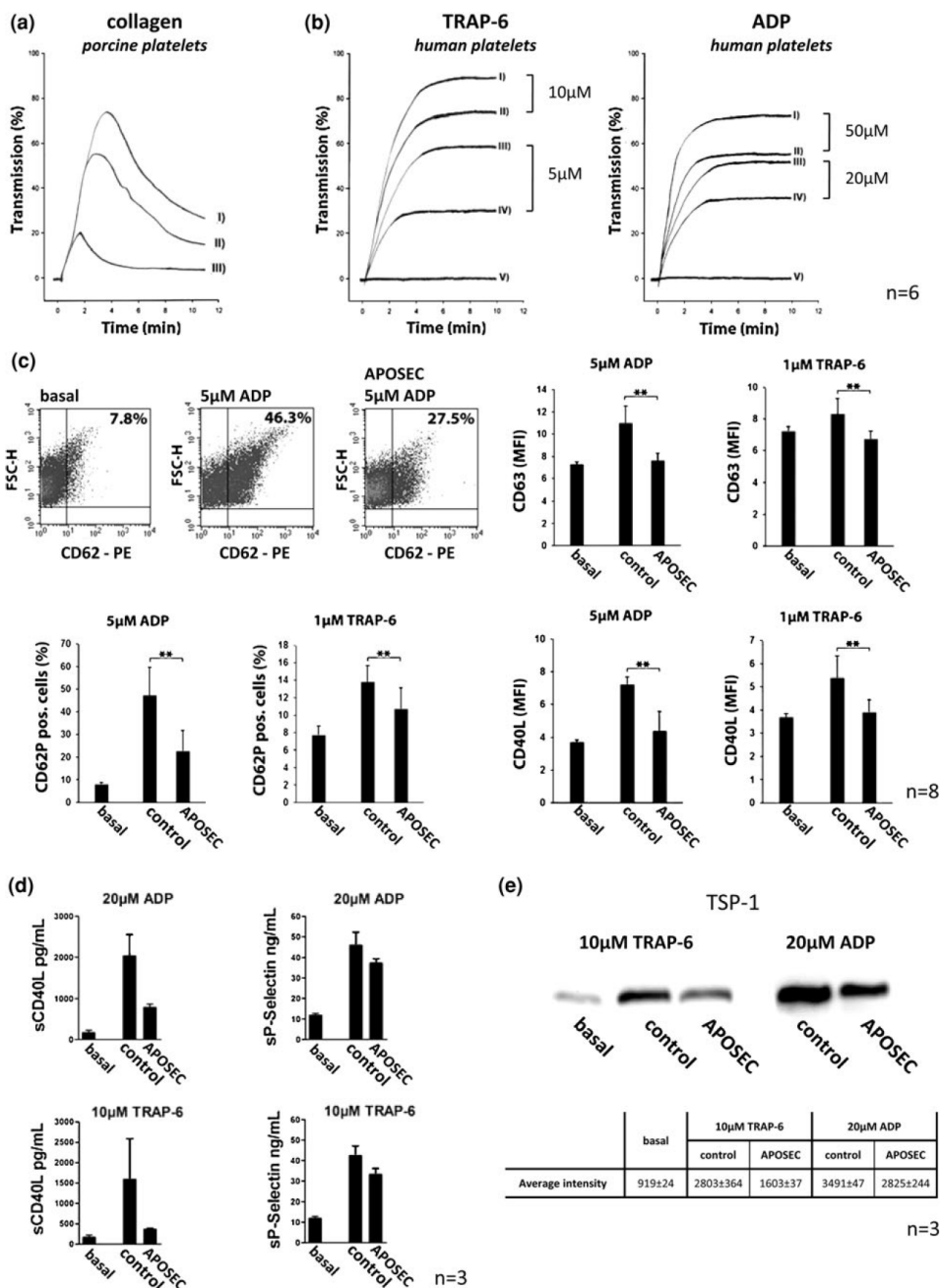
Fig. 3 Influence of APOSEC on platelet aggregation and activation. Aggregation experiments are depicted in a: I collagen (10 µg/mL); II collagen (10 µg/mL) + APOSEC 1×10^6 ; III collagen (10 µg/mL) + APOSEC 1×10^7 , b: left: I TRAP-6 (10 µM); II TRAP-6 (10 µM) + APOSEC 2×10^5 ; III TRAP-6 (5 µM); IV TRAP-6 (5 µM) + APOSEC 2×10^5 ; V basal/APOSEC 2×10^5 , right: I ADP (50 µM); II ADP (50 µM) + APOSEC 2×10^5 ; III ADP (20 µM); IV ADP (20 µM) + APOSEC 2×10^5 ; V basal/APOSEC 2×10^5 , c: surface exposure of CD62P, CD63, and CD40L after stimulation with ADP or TRAP-6 in the presence or absence of APOSEC. Influence of APOSEC on secreted activation markers is shown in d and e. Levels of sCD40L, sP-selectin, and thrombospondin-1 were lower in the supernatant of APOSEC treated platelets when compared to controls

could be confirmed in double-blind randomized trials which have led to a class IIA recommendation of use of anti GP IIb/IIIa in the ACC/AHA guidelines [5, 46].

Currently there is no standard in measuring microvasculature dysfunction in vivo. Several techniques including coronary angiography, contrast echocardiography, and MRI are used clinically and experimentally to describe MVO. Each of these techniques measures slightly different biological and functional parameters [9]. We therefore decided to confirm our MRI data with cardiac catheterization measurements. A low TIMI frame count indicates a sufficient blood flow in the small vessels; on the other hand a high TIMI frame count is associated with microvascular occlusion [18]. The angiographic myocardial blush grade is a standard method to clinically assess myocardial tissue perfusion [24]. It has a direct impact on patients' prognosis since a persistently abnormal myocardial blush grade was shown to result in reduced functional parameters in the long-term [30].

No-reflow phenomenon is known to be associated with persistent ST-elevation and ventricular arrhythmias [12, 31]. About 25 % of patients ST-segment abnormalities persist even though coronary blood flow has been restored. Therefore, we sought to determine whether APOSEC has an effect on ECG alterations during AMI. As shown in Table 3, infusion of APOSEC led to a normalization of ST segment alterations in the majority of treated animals. In addition, arrhythmic episodes were lower in the APOSEC group during occlusion and reperfusion.

The beneficial effects of APOSEC on MVO in our porcine in vivo AMI model are in line with in vitro data obtained after exposure of porcine platelets to APOSEC. Co-incubation of platelets with APOSEC prevented platelet aggregation triggered by collagen. Based on these findings further experiments were performed with human platelets and similar effects could be observed. The addition of TRAP-6 at a final concentration of 10 µM and ADP at a concentration of 50 µM caused platelets to fully aggregate and this aggregation was effectively impaired by preincubation of platelets with APOSEC. Interestingly, APOSEC derived from PBMCs isolated from diabetic and



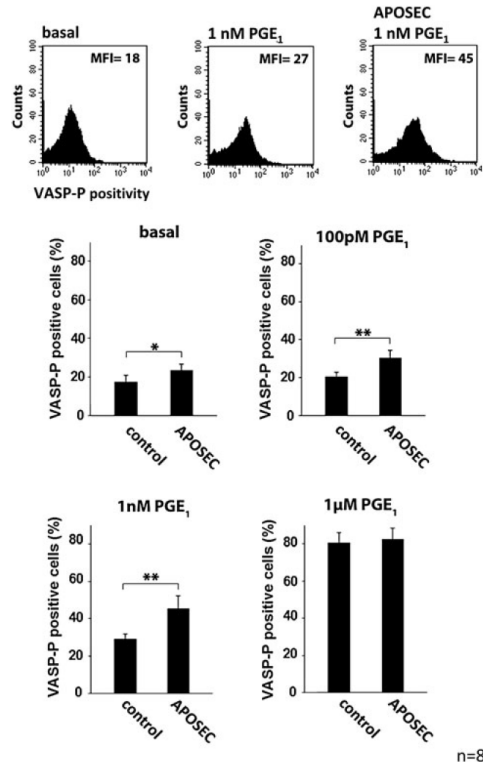


Fig. 4 In vitro effect of APOSEC on VASP-phosphorylation. Platelets from healthy donors in the presence and absence of APOSEC were analyzed for basal and PGE₁ induced VASP-phosphorylation. Co-incubation with APOSEC led to significantly increased levels of intraplatelet phosphorylated VASP

heart insufficiency patients triggered the same effects compared to APOSEC obtained from healthy patients.

Platelet surface P-selectin is considered to be the “gold standard” marker of platelet activation and was significantly reduced after preincubation of purified platelets with APOSEC [43]. This finding was further supported by reduced platelet surface markers CD63 and CD40L and lower concentrations of sCD40L, sP-selectin, and TSP-1 in the supernatant of APOSEC exposed platelets.

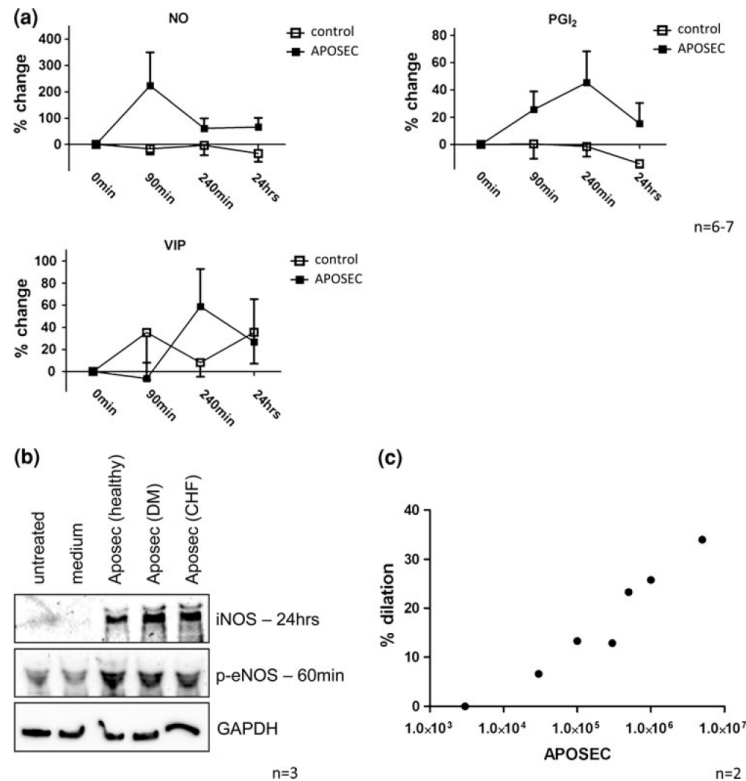
A recent paper by Köhler et al. [35] has provided profound evidence that the phosphorylation state of VASP is crucially important for the extent of myocardial ischemia/reperfusion injury. Increased intra-platelet phosphorylated VASP was shown to prevent platelet activation and platelet-neutrophil complex formation during AMI. These findings were meticulously confirmed with VASP knock-out animals, bone marrow chimeric animals and a platelet

transfer model. Therapeutic augmentation of phosphorylated VASP using a guanylyl cyclase activator was shown to be effective in a rodent animal model [54]. We consequently addressed the question whether APOSEC is capable to induce VASP phosphorylation in platelets. Indeed, we were able to show that APOSEC led to an increase of phosphorylated VASP, and these effects of APOSEC could be observed in the absence as well as in the presence of submaximal effective concentrations of prostaglandin E₁.

Besides platelet activation and aggregation, endothelial dysfunction in the small coronary vasculature is another major component in the pathophysiology of the no-reflow phenomenon. During reperfusion the endothelium is injured by oxygen free radicals resulting in an impaired endothelium-dependent vasodilation [55]. Besides, aspirates from coronary arteries obtained during PCI were shown to contain vasoconstrictor factors [34]. The concept of increased vasomotor tone in the area of MVO is supported by several clinical trials, testing different vasodilators during PCI. Currently, adenosine, verapamil or nitroprusside are a recommended therapeutic option for the treatment of no-reflow [25, 33]. Since “classical” vasodilatory drugs have been proven beneficial in the setting of no-reflow, we investigated whether APOSEC has also an effect on the vasomotor tone. In plasma samples obtained after AMI induction, systemic levels of vasodilatory mediators were heightened. In line with this finding we were able to show that HUVEC upregulated iNOS and p-eNOS expression after application of APOSEC. Besides these long-term effects on NO synthases, also a direct vasodilatory impact of APOSEC on isolated coronary vessel rings was observed, which was independent of NOS activity (Fig. 5, Suppl. Fig. 2).

Despite the effects of APOSEC on expression and activation of nitric oxide synthases, some immediately occurring effects might (also) be caused directly by biologically active compounds residing in APOSEC. In this regard, the identification of significant amounts of nitrite/nitrate in APOSEC preparations might be of central importance. As APOSEC is extensively dialyzed we can exclude the possibility that NO decomposition products nitrite and nitrate are present in APOSEC. Therefore, we consider it safe to conclude that protein adducts of nitric oxide represent a biologically active ingredient of APOSEC. The NO-axis has been shown to mediate cardioprotective signalling [27, 29] and locally liberated NO might be responsible for some of the immediate effects of APOSEC, especially those we could observe in experiments dealing with vascular tension and platelet activation. Specifically, such a mechanism would be in line with APOSEC-mediated vasodilation that occurs in L-NAME treated coronary rings and the finding that APOSEC enhances VASP phosphorylation even when applied alone (i.e., in the absence of prostaglandin E₁).

Fig. 5 Impact of APOSEC on vasodilation. Vasodilatory mediators (NO, PGI₂, VIP) were increased in AMI treated animals when compared to control pigs (a). HUVEC treated with APOSEC evidenced a strong induction of iNOS and p-eNOS (b, one representative experiment; *n* = 3). Myographic analysis of isolated coronary vessel rings showed a direct dose-dependent effect of APOSEC on vascular tonus (c)



For the current study APOSEC was produced and tested in allogeneic fashion, hence all experiments were performed with APOSEC obtained from (genetically non-identical) donors of the same species. In order to extend to the clinical reality we also obtained APOSEC from diabetic and heart failure patients. As shown in Suppl. Figs. 3 and 5b concentrations of reference cytokines and results of functional assays (platelet aggregation, iNOS, p-eNOS induction in HUVEC) were comparable in APOSEC derived from healthy and diseased patients. Consequently, these data suggest autologous (“auto-transplantation” of APOSEC derived from a diseased patient) as well as allogeneic source (APOSEC derived from healthy donors, similar to plasma derivatives) might be feasible options for patients suffering of hypoxia induced ischemic conditions. In respect to planned autologous and allogeneic APOSEC production strict regulatory prerequisites (e.g., virus inactivation, potency assays, and mandated GMP facilities) have to be met in order to reach human clinical trials.

Conclusion and outlook

APOSEC is a compound made of soluble factors secreted by irradiated PBMC and has previously been shown to abrogate myocardial damage in a large animal ischemia reperfusion AMI model. We have evidenced that this “biological” induces peri-infarct conditioning and homing of autologous c-kit positive cells into the hypoxic myocardium and thus prevents ventricular remodeling [39]. These data are in line with other published reports showing that secretomes derived from hematopoietic and mesenchymal stem cells display similar features in experimental ischemic conditions [13, 20, 36, 56].

In this study we present *in vivo* and *in vitro* data showing that APOSEC is able to abrogate platelet aggregation, induce vasodilation and attenuates microvascular obstruction in an experimental large animal AMI model. We believe that APOSEC combines the following favorable features (1) obtaining PBMC for APOSEC production is simple compared to stem cell based compounds; (2) minimal or no

antigenicity owing to protein-only content; (3) potentially “off the shelf” utilization in the setting of AMI. Our data on microvascular obstruction adds further support to the notion that APOSEC initiates a multiplicity of favorable (pleiotropic) effects in hypoxic conditions.

The “stem cell centric vision” (e.g., Bolli et al. [11]) in regenerative medicine is currently under critical appraisal [61] and our study underlines the credibility of the “paracrine hypothesis” [19, 36]. Good clinical manufacturing of PBMC derived APOSEC, which is currently in the planning phase, will pave the way to first human trials.

Limitation

In all in vitro and in vivo experimental settings (porcine reperfused AMI model, in vitro experiments using porcine and human cells), APOSEC was tested in a syngeneic fashion only, in order to obviate inter-species influences.

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Conflict of interest This study was funded by the Christian Doppler Research Association, APOSIENCE AG and the Medical University of Vienna. The authors declare competing financial interests. The Medical University of Vienna has claimed financial interest (Patent number: EP2201954, WO2010070105-A1, filed 18 Dec 2008). H. J. A. is a shareholder of APOSIENCE AG, which owns the rights to commercialize APOSEC for therapeutic use. All other authors declare that they have no conflict of interest.

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