Beer et al. 2015 - Analysis of the Secretome of Apoptotic Peripheral Blood Mononuclear Cells: Impact of Released Proteins and Exosomes for Tissue Regeneration

Journal Club 13.11.2017

Semmler Lorenz

Introduction to regenerative medicine

- Regenerative medicine aims to restore damaged or dysfunctional tissue
- Major advances in drug therapies, surgical interventions and organ transplantation, BUT:
- Tremendous problems remain unsolved concerning regeneration of injured organs
- Previously, the use of stem cells has shown promising results, however
- It was recently acknowledged that paracrine factors are the key
- Boosted by apoptosis

Previous studies

- Stressed peripheral blood mononuclear cells (PBMCs) could promote tissue protection and repair through paracrine activities
- Their secretome has been shown to enhance angiogenesis and wound healing *in vitro* and *in vivo* (regeneration of myocardium and brain after acute ischemic injuries)
- Only a few previous studies have identified the paracrine factors involved (IL-8, IL-16, MMP-9, VEGF…)

This present study...

- Analyzed in detail the biological components present in conditioned medium (CM)
- Looked in depth at proteins, lipids and extracellular vesicles
  - Proteins: several secreted proteins have been identified to exert cytoprotective and regenerative capacities
  - Lipids: have been shown to modulate immune function, induce angiogenesis and enhancing wound healing by upregulating pro-angiogenic proteins
  - Extracellular vesicles: intercellular interaction, (mRNA, microRNA, protein, lipid delivery; *e.g. exosomes from mesenchymal stromal cells induced neurogenesis after stroke)*

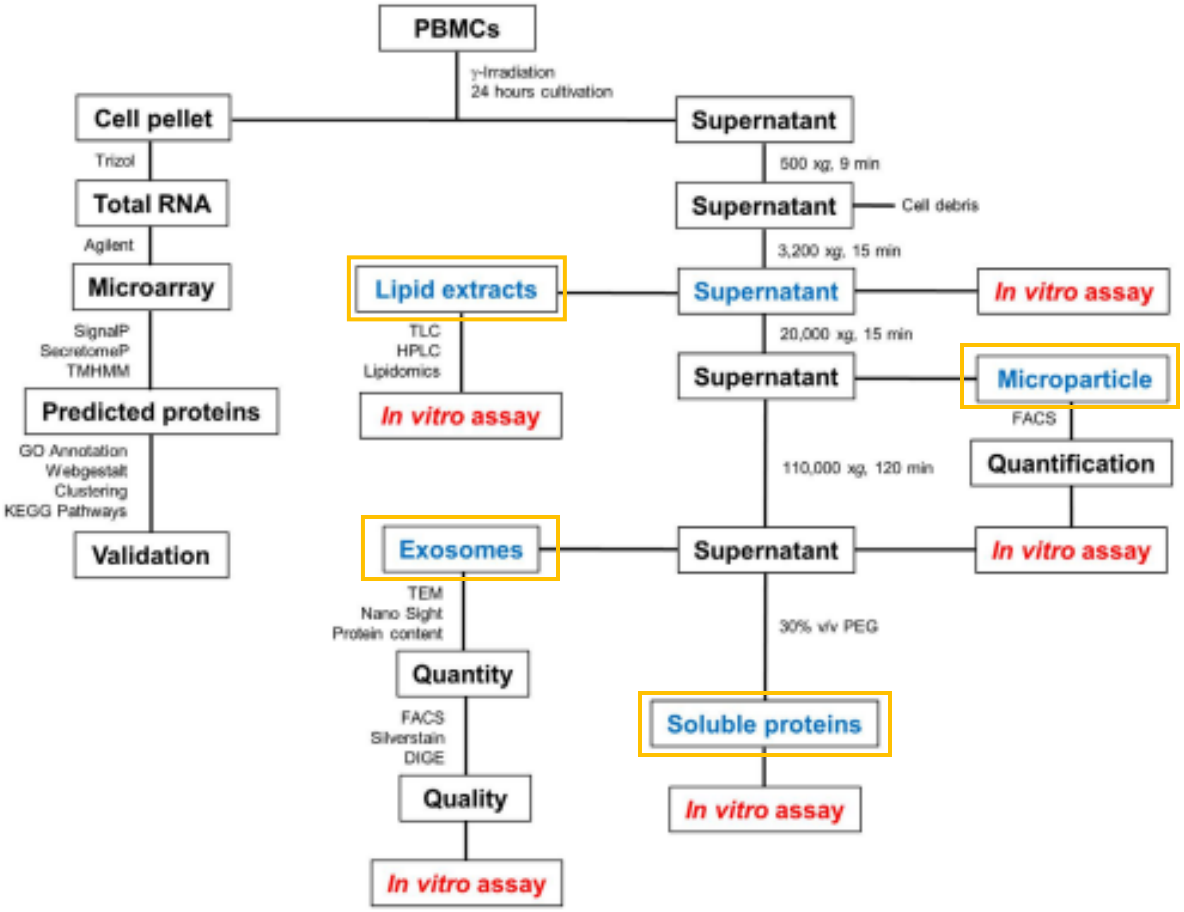
„Canapé“ Aim of the study

- Aims: Characterization of the secretome of (non-)irradiated PBMCs
  - Combination of methods, including transcriptomics, lipidomics, functional *in vitro* assays
- Evaluation of viral-cleared PBMC secretome
  - Retention of preventative potency in a porcine closed-chest-reperfusion, acute myocardial infarction (AMI) model

- Results:
  - Irradiation induced the expression of pro-angiogenic factors, the shedding of microparticles and exosomes and the production of oxidized phospholipids
  - Exosomes and proteins are the two major biologically active components present in the secretome of irradiation-induced PBMCs
  - *In vivo*: „cell free“ regenerative medicine shows potency in preventing ventricular remodeling after an experimental AMI

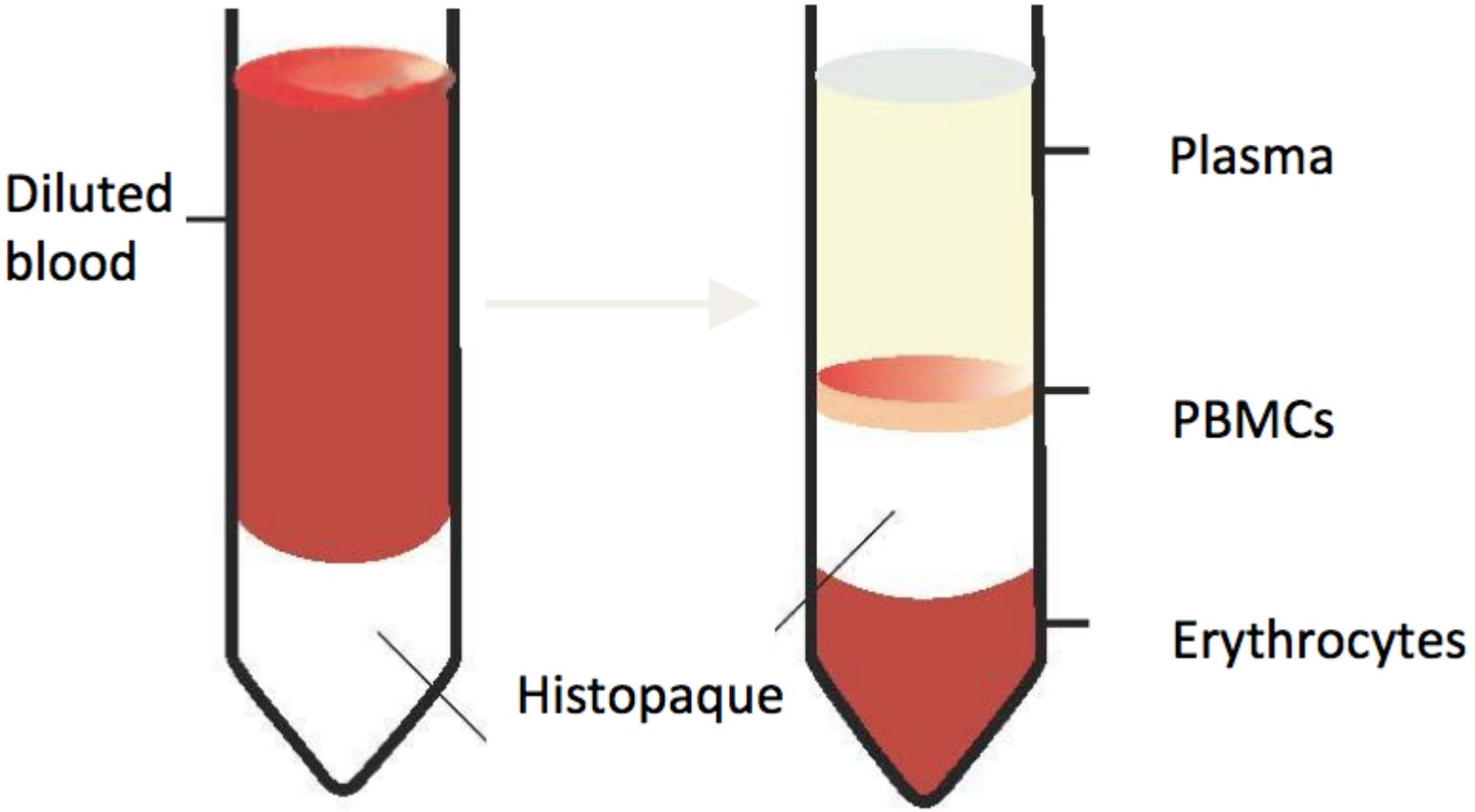
Materials and methods

Experimental workflow



Beer, L. *et al.* Analysis of the Secretome of Apoptotic Peripheral Blood Mononuclear Cells : Impact of Released Proteins and Exosomes for Tissue Regeneration. *Nat. Publ. Gr.* 1–18 (2015). doi:10.1038/srep16662

Isolation of PBMCs



http://os.bio-protocol.org/attached/image/20141121/20141121000327_5767.jpg
Accessed 11.11.2017

Results

Changes in the PBMC transcriptome

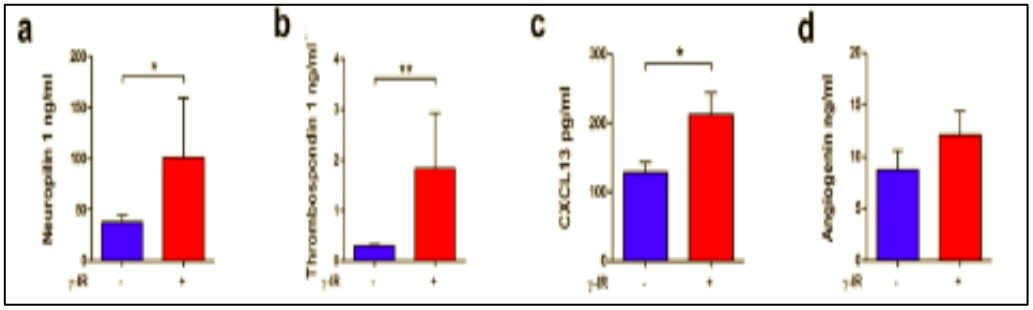
- PBMCs: irratiated vs. non-irradiated, culture for 2h/4h/20h
- RNA isolation and microarray analysis (evaluation of gene expression)

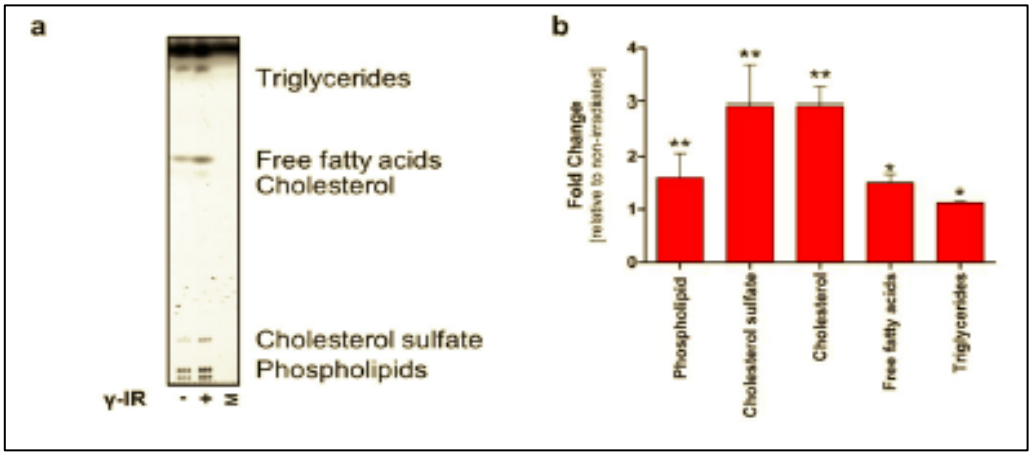
- Non-irradiated cells:
  - **167** transcripts that encoded actively secreted proteins
  - Enrichment in genes involved in **amino acid transport** and **endocrine regulation**
- Irradiated cells:
  - **213** transcripts that encoded actively secreted proteins
  - Enrichment in genes involved in **angiogenesis**, **wound healing** and **leukocyte trafficking regulation**

Changes in the PBMC transcriptome

Data suggest that gene expression is **shifted from metabolic processes** in the non-irradiated state **towards tissue regeneration** after irradiation.

Comparison of secretomes



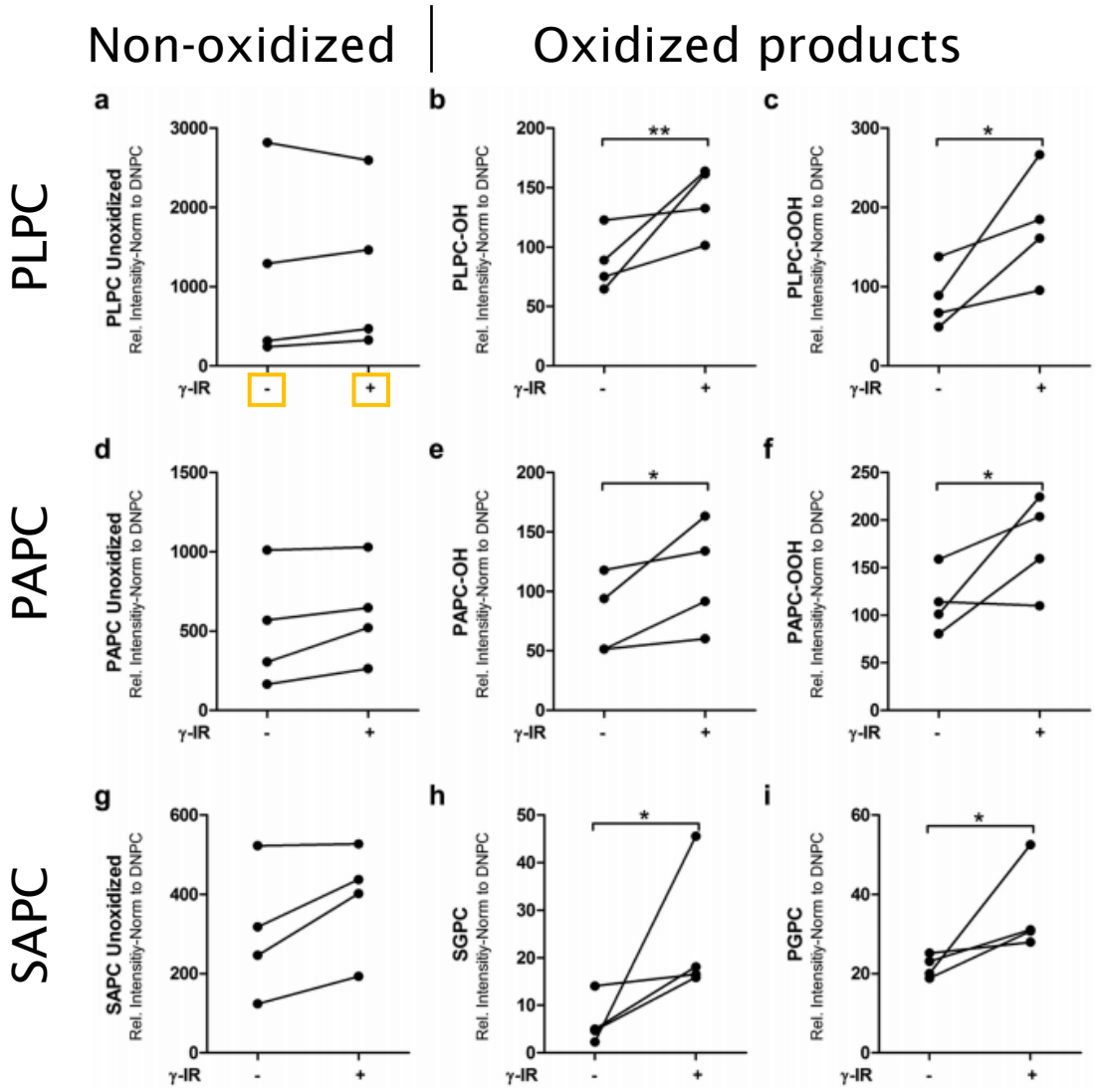


- Significantly higher concentrations of neuropilin, thrombospondin, CXCL13 and angiogenin in irradiated PBMCs secretome
- Significantly higher concentrations of phospholipids, cholesterolsulfate, cholesterol and free fatty acids in irradiated PBMCs secretome

Beer, L. *et al.* Analysis of the Secretome of Apoptotic Peripheral Blood Mononuclear Cells: Impact of Released Proteins and Exosomes for Tissue Regeneration. *Nat. Publ. Gr.* 1–18 (2015). doi:10.1038/srep16662

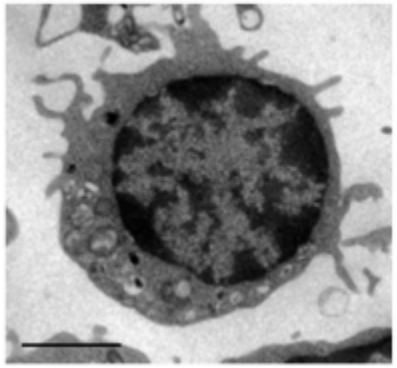
Ionizing radiation induces phospholipid oxidation

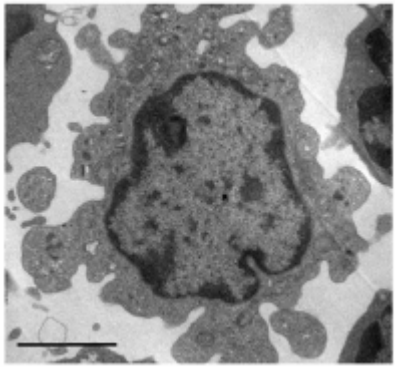
- Quantification of oxidized products in the conditioned medium (CM) of irratiated vs. non-irradiated PBMCs 20h after irradiation.
- Irradiated samples showed significantly higher concentrations of oxidized lipid products in the CM.



Beer, L. *et al.* Analysis of the Secretome of Apoptotic Peripheral Blood Mononuclear Cells: Impact of Released Proteins and Exosomes for Tissue Regeneration. *Nat. Publ. Gr.* 1–18 (2015). doi:10.1038/srep16662

Ionizing radiation induces release of microparticles





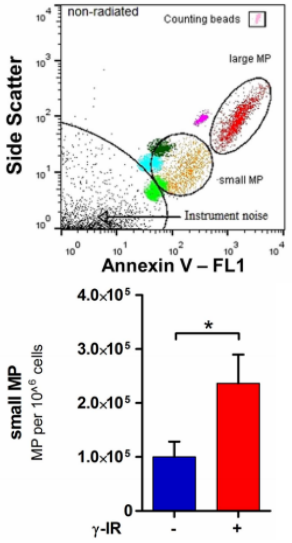
Left I Non-irradiated: largely intact plasma membrane and cell nucleus

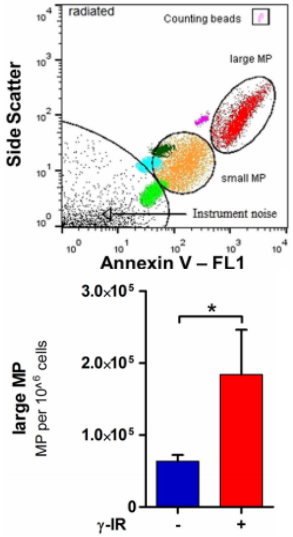
Right I Irradiated: cellular shrinking, plasma membrane fragmentation, chromatin condensation

Apoptosis is known to induce shedding of plasma membrane microparticles.

Isolation of microparticles (MP) from CM at 20h after irradiation.
Stain with annexin V (apoptosis marker).
Typical MP range: 0.2 – 1.0 µM

Irradiation induced the release of both small and large MPs.



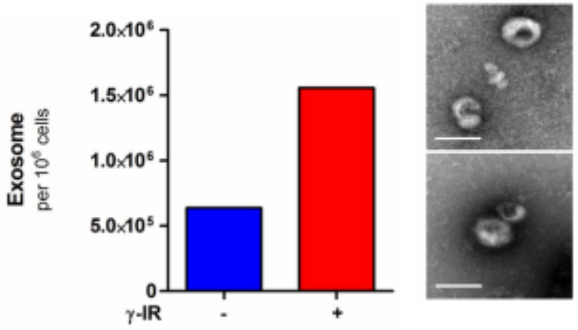


Beer, L. *et al.* Analysis of the Secretome of Apoptotic Peripheral Blood Mononuclear Cells : Impact of Released Proteins and Exosomes for Tissue Regeneration. *Nat. Publ. Gr.* 1–18 (2015). doi:10.1038/srep16662

Ionizing radiation induces release of exosomes and modulates protein content

Transmission electron microscopy (TEM)

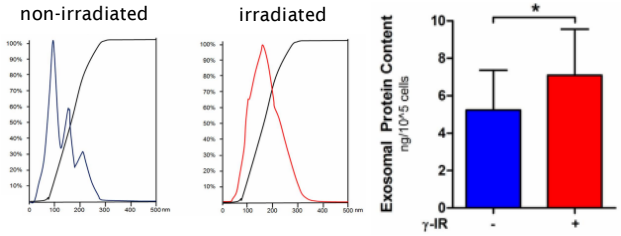
- Isolation of exosomes from CM of irradiated / non-irradiated PBMCs at 20h
- 3x more exosomes in CM of irradiated PBMCs



Beer, L. *et al.* Analysis of the Secretome of Apoptotic Peripheral Blood Mononuclear Cells: Impact of Released Proteins and Exosomes for Tissue Regeneration. *Nat. Publ. Gr.* 1–18 (2015). doi:10.1038/srep16662

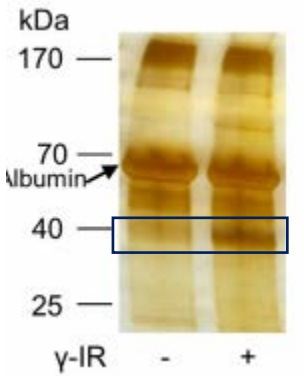
FACS (cell sorting) analysis

- Exosome markers: CD63, CD9
- Different size of exosomes (143 nm ± 56 nm non-irradiated / 177 nm ± 63 nm irradiated)
- Higher total protein content in exosomes in irradiated cells

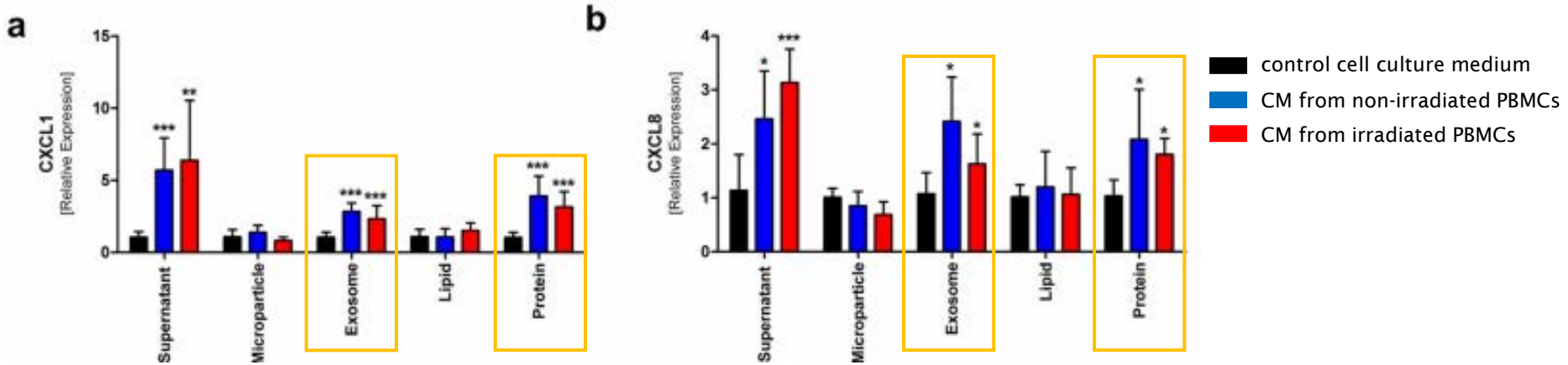


SDS-PAGE / 2D gel electrophoresis

- Proteins: seperated on SDS-PAGE, stained with silver stain
- Differentially expressed proteins in exosomes of irratiated vs. non-irradiated PBMCs



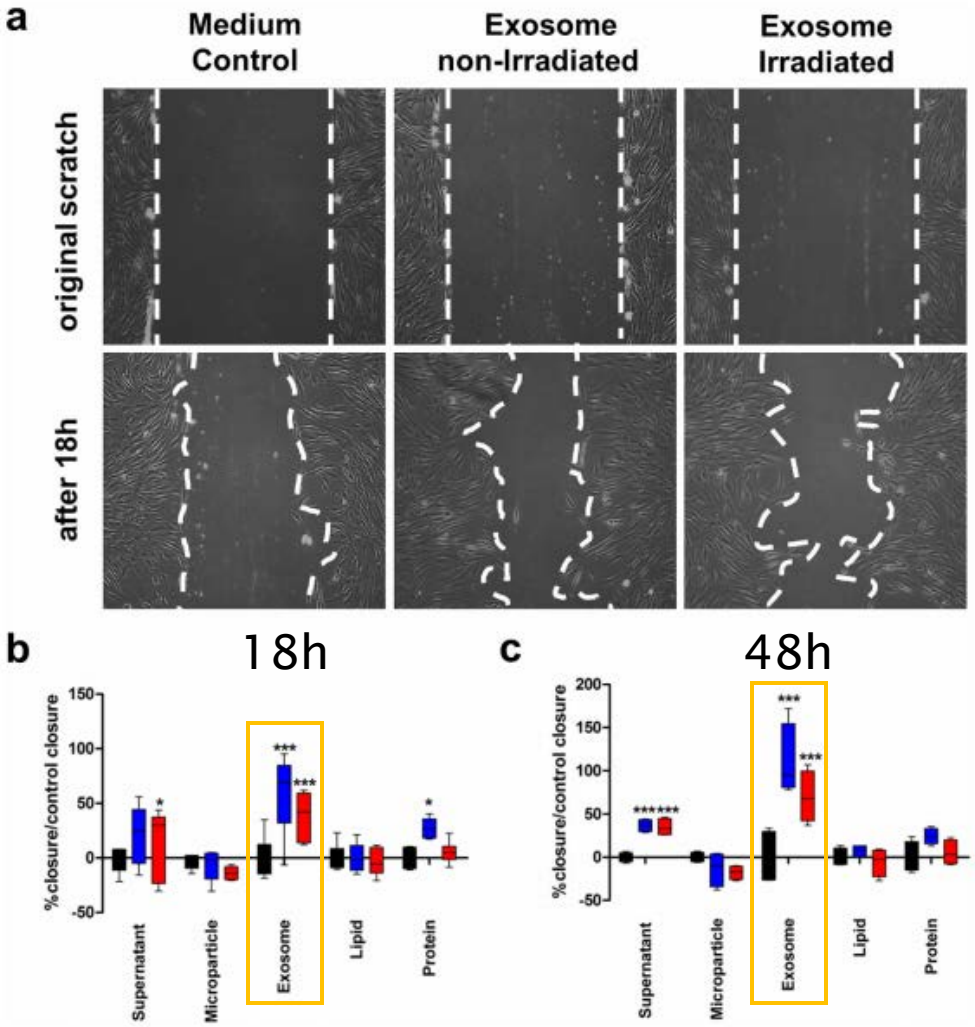
Exosomes and proteins stimulate CXCL1 and CXCL8 expression



- CXCL1 and CXCL8 responses in fibroblasts are induced after stimulation with total CM.
- CXCL1: growth factor, signal for inflammation
- CXCL8: = IL-8, mediator in inflammation and angiogenesis
- Expression of both is increased when stimulated with exosomes or proteins from irradiated and non-irradiated PBMCs.
- Data from fibroblasts, similar results in keratinocytes

Beer, L. *et al.* Analysis of the Secretome of Apoptotic Peripheral Blood Mononuclear Cells: Impact of Released Proteins and Exosomes for Tissue Regeneration. *Nat. Publ. Gr.* 1–18 (2015). doi:10.1038/srep16662

Proteins and exosomes in conditioned media (CM) are biologically active



- Scratch assay of fibroblast monolayers (similar results in keratinocytes)
- Untreated or treated with exosome preparations from non-irradiated or irradiated PBMCs.
- Treatment with PMBC-derived exosomes accelerated wound closure.
- No detectable difference between irradiated and non-irradiated PBMCs.
- Exosomes (and proteins) are the main biologically active component of the CM.

Beer, L. *et al.* Analysis of the Secretome of Apoptotic Peripheral Blood Mononuclear Cells: Impact of Released Proteins and Exosomes for Tissue Regeneration. *Nat. Publ. Gr.* 1–18 (2015). doi:10.1038/srep16662

Description of the viral-cleared, GMP-compliant supernatant used in the *in vivo* AMI

- Comparison in domestic pig closed-chest reperfusion acute myocardial infarction model
- Quantity and quality of biological components were comparible between GMP-compliant and experimentally prepared supernatants
- GMP-compliant CM enriched in oxidized phospholipids
- No microparticles (filtered through 0,2 µm filter)
- Comparable capacity to attenuate ischemic damage, improved cardaic output and reduced infarct areas
- First proof of principle

	Parameters	Medium Control (n = 7)	CM ($1,5 \times 10^6$; n = 4)	CM + PR ($1,5 \times 10^6$; n = 6)	
After 3 days	Weight (kg)	31.9 ± 0.9	32.0 ± 1.2	34.2 ± 0.5	n.s.
	Age (days)	90 ± 0	90 ± 0	90 ± 0	n.s.
	LVEDV (ml)	67.6 ± 2.8	75.6 ± 2.2	83.0 ± 4.0*	*
	LVESV(ml)	38.4 ± 2.5	47.4 ± 1.7*	48.7 ± 4.3	n.s.
	LVSV (ml)	29.2 ± 1.3	28.3 ± 2.2	34.3 ± 2.0*	n.s.
	LVEF (%)	43.4 ± 1.9	37.3 ± 2.2	41.7 ± 2.9	n.s.
	HR/min	111 ± 6	87 ± 9*	77 ± 3**	**
	CO (l/min)	3.2 ± 0.1	2.4 ± 0.1*	2.6 ± 0.2*	**
	CI (l/min/m2)	3.6 ± 0.1	3.1 ± 0.3	3.3 ± 0.2	n.s.
	Infarct %	18.2 ± 1.7	13.1 ± 2.8	12.3 ± 1.9*	n.s.
After 30 days	Weight (kg)	39.4 ± 0.5	50.0 ± 1.8***	55.7 ± 0.7***	***
	Age (days)	120 ± 0	120 ± 0	120 ± 0	n.s.
	LVEDV (ml)	54.8 ± 4.1	107.5 ± 6.7***	102.5 ± 6.0***	***
	LVESV(ml)	32.9 ± 4.0	65.5 ± 3.4***	53.9 ± 4.3**	***
	LVSV (ml)	21.8 ± 1.8	42.0 ± 4.4**	48.6 ± 2.9***	***
	LVEF (%)	40.5 ± 3.6	38.9 ± 2.3	47.6 ± 2.1	n.s.
	HR/min	114 ± 7	123 ± 5	109 ± 3	n.s.
	CO (l/min)	2.4 ± 0.1	5.1 ± 0.4***	5.3 ± 0.3 ***	***
	CI (l/min/m2)	2.5 ± 0.1	5.0 ± 0.3***	4.7 ± 0.3***	***
	Infarct %	12.6 ± 1.4	9.8 ± 0.6	8.2 ± 1.7	n.s.

Beer, L. *et al.* Analysis of the Secretome of Apoptotic Peripheral Blood Mononuclear Cells: Impact of Released Proteins and Exosomes for Tissue Regeneration. *Nat. Publ. Gr.* 1–18 (2015). doi:10.1038/srep16662

Discussion, conclusion and summary

Identification of proteins in the secretome

- Bioinformatics-based analysis of the secretome
- Revealed 213 upregulated proteins in response to irradiation
- Involved biological processes related to **angiogenesis, cell proliferation and cytokine signaling**
- *Examples*: adrenomedullin, MMP9, VEGFA, TIMP-1
- *growth differentiation factor 15, insulin like growth factor*

- Purified CM proteins induced:
  - Cell migration
  - CXCL1 and CXCL8 expression (in FBs and KCs) → **involved in wound healing and angiogenesis**

Presence and activity of extracellular vesicles in PBMC-derived CM

- Importance of extracellular vesicles as mechanism of intercellular communication
- Stimulate regenerative capacity of injured tissues (increase endothelial cell proliferation, induce angiogenesis, modulate extracellular matrix interactions)
- Exact mechanism unclear
- Exosomes and proteins are the two main biological components that stimulate CXCL1 and CXCL8 gene expression

Effect of irradiation on the oxidized lipid content of the secretome

- Focus on the oxidized phosphatidylcholines (oxPCs)
- Irradiation promoted the formation of oxidized lipid species
- Pro-angiogenic and immunomodulatory properties
- **CM obtained from irradiated PBMCs contained significantly higher concentrations of specific oxPCs**
- oxPCs are known to induce expression of CXCL8, modulate angiogenesis
- No detectable effect *in vitro*

Outlook and questions for the future

Thank you for your attention!