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Chapter 16

Up-Regulation of Autophagy Defense Mechanisms in Mouse Mesenchymal Stromal Cells in Response to Ionizing Irradiation Followed by Bacterial Challenge

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Additional information is available at the end of the chapter

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1. Introduction

Mesenchymal stroma along with epithelium, endothelium, reticuloendothelium, and lymphoid components is an essential constituent of tissue barriers that sustain immunochemical homeostatic interactions of tissue with internal and external environments. Thus, mesenchymal stroma protects the body from infections [1-8]. A breach of immune and structural integrity of tissue barriers under patho-physiological conditions such as complicated injury can lead to translocation of bacteria from different host-associated microbiomes and colonization of vital organs that can ultimately result in multiple organ failure and sepsis [9].

It is well documented that suppression of radiosensitive lymphoid and epithelial cells by ionizing irradiation results in impairment of tissue barriers and provokes bacterial translocation and sepsis leading to lethal outcome [10-12]. Under these circumstances one would expect increasing stress impact to the ubiquitously present and relatively radioresistant mesenchymal stromal components and their implication in host defense response [13]. This idea is supported by experimental and clinical observations indicating that injury can induce recruitment of mesenchymal stromal cells (MSCs) from bone marrow and promote their proliferative activity in order to re-constitute integrity of fractured tissue and mediate natural debridement [2, 5, 14, 15]. Moreover, it has been shown recently that transplanted MSCs can suppress experimental sepsis and can promote healing of radiation-induced cutaneous injury and survival from acute radiation syndrome [16-20]. All of this evidence suggests that MSCs play a crucial role in mitigation of systemic and local effects of tissue injury under different pathophysio-

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logical conditions. However, in the case of total body irradiation, the dynamics of MSC response to inflammatory stimuli can be skewed due to the cytotoxic effects of irradiation; but the mechanisms of remodeling of irradiated MSCs and their antimicrobial barrier capacity are not known and need to be delineated. *In vivo* assessment of stromal cell responses against bacteria is nearly impossible, because of (i) complexity of the architecture of the mesenchymal network in tissues and (ii) the fact of lethal complications in the hematopoietic radiation syndrome occurring at so low a level of microorganisms that the responses are difficult to detect [3,10].

From this perspective our attention was attracted by the macroautophagy-lysosomal (autolysosomal) mechanism described recently *in vitro* in cultured mesenchymal fibroblastic stromal cells [13]. The autophagy/autolysosomal mechanism mediates cell secretory functions and biodegradation mechanisms implicated in phagocytosis and cell remodeling activated in response to damage to cell constituents, endoplasmic reticular (ER) stress, and protein misfolding [21-23]. Thus, the autolysosomal pathway is responsible for decomposition of damaged proteins and organelles as well as phagocytized bacteria and viruses and is considered to be a part of the innate defense mechanism [23- 25].

The dynamics of macroautophagy (hereafter referred to as autophagy) in mammalian cells are well described in recent reviews [22, 26-28]. It has been proposed that autophagy is initiated by the formation of the phagophore, followed by a series of steps, including the elongation and expansion of the phagophore, closure and completion of a double-membrane autophagosome (which surrounds a portion of the cytoplasm), autophagosome maturation through docking and fusion with an endosome (the product of fusion is defined as an amphisome) and/or lysosome (the product of fusion is defined as an autolysosome), breakdown and degradation of the autophagosome inner membrane and cargo through acid hydrolases inside the autolysosome, and, finally, release of the resulting macromolecules through permeases [22]. These processes, along with the drastic membrane traffic, are mediated by factors known as autophagy-related proteins (i.e., ATG-proteins) and the lysosome-associated membrane proteins (LAMPs) that are conserved in evolution [29]. The autophagic pathway is complex. To date there are over 30 ATG genes identified in mammalian cells as regulators of various steps of autophagy such as cargo recognition, autophagosome formation, etc. [22, 30]. The core molecular machinery is comprised of (i) components of signaling cascades, such as the ULK1 and ULK2 complexes and class III PtdIns3K complexes, (ii) autophagy membrane processing components such as mammalian Atg9 (mAtg9) that contributes to the delivery of membrane to the autophagosome as it forms, and (iii) two conjugation systems: the microtubule-associated protein 1 (MAP1) light chain 3 (i.e., LC3) and the Atg12–Atg5–Atg16L complex. The two conjugation systems are proposed to function during elongation and expansion of the phagophore membrane [22, 27, 30]. A conservative estimate of the autophagy network counts over 400 proteins, which, besides the ATG-proteins, also including stress-response factors, cargo adaptors, and chaperones such as p62/SQSTM1 and heat shock protein 70 (HSP70) [23, 27, 30, 32, 33-35].

Autophagy is considered as a cytoprotective process leading to tissue remodeling, recovery, and rejuvenation. However, under circumstances leading to mis-regulation of the autolyso-

somal pathway, autophagy can eventually cause cell death, either as a precursor of apoptosis in apoptosis-sensitive cells or as a result of destructive self-digestion [36].

We hypothesized that: (i) MSCs enable activation of the autophagy pathway in response to ionizing irradiation; (ii) this mechanism is a part of adaptive remodeling essential for recovery of MSCs from the radiation-induced injury; and (iii) activation of autophagy in the irradiated MSCs can be potentiated by a challenge with Gram-negative or Gram-positive bacteria, e.g., *Escherichia coli* or *Staphylococcus epidermidis,* in order to sustain the MSC phagocytic antibacterial functions. The objective of the current chapter is to provide evidence to substantiate the proposed hypothesis.

2. Hypothesis test: Experimental procedures and technical approach

2.1. Mouse bone marrow Mesenchymal Stromal Cells (MSCs)

The cultures of MSCs were established and expanded as described previously [13]. Phenotype, proliferative activity, and colony-forming ability of the cells were monitored by flow cytometry and immunofluorescence imaging using positive markers for MSCs, i.e., CD44 and Sca1 [13].

2.2. Irradiation of MSCs and challenge with bacteria

2.2.1. MSC irradiation

MSC irradiation with gamma-photons was conducted using the ^{60}Co source in the Armed Forces Radiobiology Research Institute. The range of the applied doses was from 1 Gy through 12 Gy at a dose rate of 0.4 Gy/min. Dosimetry was performed using the alanine/electron paramagnetic resonance system. Calibration of the dose rate with alanine was traceable to the National Institute of Standards and Technology and the National Physics Laboratory of the United Kingdom. The irradiated cells were given a 24 h rest and then were subjected to either analyses or a challenge with *S. epidermidis* or *E. coli.*

2.2.2. Challenge of MSCs with bacteria

Irradiated and non-irradiated MSC cultures (~90% confluency) were challenged with either *S. epidermidis* or *E. coli* ($5x10^7$ bacteria/ml) for 1-3 h in antibiotic-free medium. For assessment of the cellular alteration during a period ≥ 3 h, the incubation medium was replaced with fresh medium containing penicillin and streptavidin antibiotics.

2.3. Cell analyses

Cell analysis for (i) the radiation-induced DNA double-strand breaks, viability, pro-apoptotic alterations, MSC proliferative activity, integrity of cell monolayers, and colony-forming activity; (ii) bacterial growth suppression, (iii) bacterial phagocytosis and autophagy (ATG), and (iv) response of stress-proteins, were conducted using flow cytometry techniques,

fluorescence confocal imaging, protein immunoblotting, bright-field microscopy, and transmission electron microscopy (TEM).

The flow cytometry–based assessments of (i) the radiation-induced DNA double-strand breaks; (ii) proliferative activity; and (iii) cell viability were conducted using, respectively, the H2A.X phosphorylation assay kit (Cell Signaling Solutions, Temecula, CA); Click-iT® EdU Cell Proliferation Assay Kit, which utilizes a modified nucleoside, EdU (5-ethynyl-2′-deoxyuridine) that, in turn, is incorporated during *de novo* DNA synthesis in a quick-click chemistry reaction] (Life Technologies Corp., Grand Island, NY); and the CYTOX® Blue stain (Life Technologies Corp., Grand Island, NY).

The radiation-induced apoptotic response in MSCs was determined by immunoblot analysis of caspase-3, a marker of apoptosis.

The data presented in Fig. 1 indicate that the MSC cultures displayed a high integrity and survival from damage produced by irradiation with doses 1 Gy – 12 Gy. The irradiated cells challenged with bacteria were also able to sustain integrity of confluent monolayers (Fig. 1). The treated cells did not manifest signs of pro-apoptotic alterations. Moreover, MSCs challenged with *E. coli* and *S. epidermidis* at 24 h following irradiation (8 Gy) were able to suppress the bacterial growth by 1.4-fold and 1.8-fold, respectively (not shown).

2.4. Analysis of the cell proteins

Proteins from MSCs were extracted in accordance with the protocol described previously [12]. Aliquots of proteins were resolved on SDS-polyacrylamide slab gels (NuPAGE 4-12% Bis-Tris; Invitrogen, Carlsbad, CA). After electrophoresis, proteins were blotted onto a PDVF membrane and the blots were incubated with antibodies (1 μg/ml) raised against MAP LC3, Lamp1, p65(NFκB), HSP70, Sirt3a, SUMO1, and actin (Abcam, Santa Cruz Biotechnology Inc., LifeSpan Biosciences, Inc., eBiosciences) followed by incubation with species-specific IgG peroxidase conjugate.

2.5. Immunofluorescent staining and image analysis

MSCs (5 specimens per group) were fixed in 2% paraformaldehyde, processed for immunofluorescence analysis and analyzed with fluorescence confocal microscopy (30). Normal donkey serum and antibody were diluted in phosphate-buffered saline (PBS) containing 0.5% BSA and 0.15% glycine. Any nonspecific binding was blocked by incubating the samples with purified normal donkey serum (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) diluted 1:20. Primary antibodies were raised against MAP LC3, Lamp1, p62/SQSTM1, p65(NFκB), FoxO3a, Tom 20. That was followed by incubation with secondary fluorochrome-conjugated antibody and/or streptavidin-AlexaFluor 610 conjugate (Molecular Probes, Inc., Eugene OR), and with Heochst 33342 (Molecular Probes, Inc., Eugene OR) diluted 1:3000. Secondary antibodies used were AlexaFluor 488 and AlexaFluor 594 conjugated donkey IgG (Molecular Probes Inc., Eugene OR). Negative controls for nonspecific binding included normal goat serum without primary antibody or with secondary antibody alone. Five confocal fluorescence and DIC

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images of crypts (per specimen) were captured with a Zeiss LSM 710 microscope. The immunofluorescence image analysis was conducted as described previously [12].

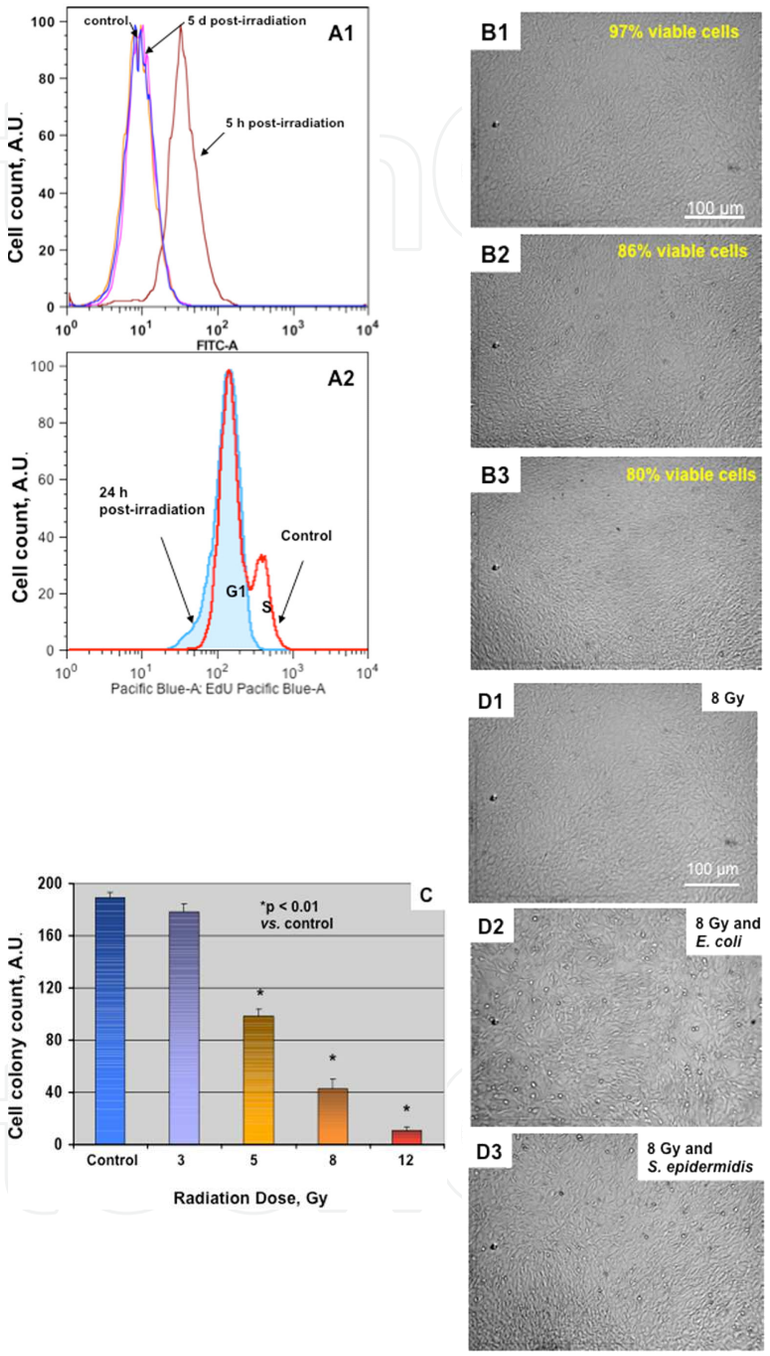


Figure 1. Functional ability of MSCs subjected to ionizing irradiation and bacterial challenge. A1. Analysis of radiation-induced DNA double-strand breaks with flow cytometry assay of the phosphorylated H2A.X (γ-H2A.X). Conditions: Control (in blue line) and irradiated MSCs were analyzed at 5 h (brown line) and 5 d (pink line) after 8-Gy irradiation. A2. Analysis of the radiation-induced suppression of MSC proliferative activity with flow cytometry assay of incorporated EdU, a modified nucleoside. The S-phase cell population was absent after irradiation Conditions: Control (red line) and irradiated (8 Gy, blue line) MSCs were analyzed 24 h after 8-Gy irradiation. A.U. is % of maximal cell count per channel. B. Bright-field microscopy analysis of effect of ionizing irradiation on ability of MSCs to form confluent

monolayers. Panel B1, control; Panel B2, 8-Gy irradiation; Panel B3, 12-Gy irradiation. Conditions: Images were captured 24 h after irradiation. Panel C. Analysis of the radiation-induced suppression of MSC colony-forming ability. Conditions: MSCs were harvested 24 h after irradiation and 200 MSCs from each radiation dose sample were aliquoted to Petri dishes and cultivated for 10 days. Panels D. Bright-field microscopy analysis of effects of bacterial challenge on ability of the irradiated MSCs shown in panel "B2" to form confluent monolayers. Panel D1 is after irradiation only (8 Gy), Panel D2 is same as "Panel D1" but after challenge with *E. coli*; Panel D3 is same as "Panel D1" but after challenge with *S. epidermidis.* Conditions: Images were captured 24 h after the bacterial challenge.

2.6. Transmission Electron Microscopy (TEM)

MSCs in culture were fixed in 4% formaldehyde and 4% glutaraldehyde in PBS overnight, post-fixed in 2% osmium tetroxide in PBS, dehydrated in a graduated series of ethanol solutions, and embedded in Spurr's epoxy resin. Blocks were processed as described previously [12,13]. The sections of embedded specimens were analyzed with a Philips CM100 electron microscope.

2.7. Statistical analysis

Statistical significance was determined using Student's *t*-test for independent samples. Significance was reported at a level of $p<0.05$.

3. Role of autophagy in adaptive response of MSCs to radiation injury and phagocytosis of *S. epidermidis* and *E. coli*

3.1. Alterations in the MSC stress-response-proteins following irradiation and bacterial challenge

The 8-Gy irradiation resulted in substantial DNA double-strand breaks in MSCs detectable with the γ-H2AX assay at 5 h post-exposure (Fig. 1A, brown line). This effect disappeared at 5 d post-irradiation recovery (Fig. 1A1, pink line). The observed DNA damage was accompanied by suppression of the cell proliferative activity determined with Click-iT® EdU Cell Proliferation Assay. As shown in Fig. 1A2 (red line), in control groups the cell populations were represented by the cells in both G1 and S phases. Following irradiation, the entire cell population was in G1 phase. The data presented in Fig. 1B indicate that the MSC cultures displayed a high integrity and survival from damage produced by irradiation at doses ranging from 1 Gy to 12 Gy, but that their ability to form colonies was reduced in a radiation dose-dependent manner (Fig. 1C).

The irradiated cells challenged with bacteria were also able to sustain integrity of confluent monolayers (Fig. 1D). These cells did not manifest signs of pro-apoptotic alterations. Moreover, MSCs challenged with *E. coli* and *S. epidermidis* 24 h after irradiation (8 Gy) were able to suppress the bacterial growth by 1.4-fold and 1.8-fold, respectively (not shown).

The data presented in Fig. 1 indicate that, despite radiation-produced damage and suppression of proliferative activity, MSCs demonstrated substantial radioresistance and absence of significant apoptotic and necrotic transformations in a wide range of radiation doses, i.e., 1-12

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Gy. Interactive investigation of the stress-response factors implicated in cell survival may be important for the development of effective therapies for radiation injury (RI).

According to a current paradigm, the general stress responses involve conserved signaling modules that, in turn, are interconnected to the cellular adaptive mechanisms [37]. It is suggested that the stress due to molecular and organelle damage, impact of pro-oxidants, and infections triggers a cascade of responses attributed to specific sensitive transcriptional and post-transcriptional mechanisms mediating inflammation, antioxidant response, adaptation, and remodeling [36-40]. Ionizing radiation (IR) *per se* stimulates signaling cascades mediated by transcription factors and pathways that are believed to play a central role in protective response(s) to the molecular and subcellular damage and the oxidative stress. They include (but are not limited to) a battery of thiol-containing redox-response elements, redox-sensitive transcription factors such as nuclear factor-kappa B (NFκB) and forkhead box O3a (FoxO3a), stress-response adaptors such as the chaperone heat-shock protein 70 (HSP70) and NAD^+-dependent deacetylase sirtuin-3 (Sirt3), and activators of the autolysosomal degradation. Overall, these effector systems are crucial in maintaining homeostasis, which is altered due to damage to the cell constituents [33, 40-46]. It should be noted that, while the role of the IR-induced NFκB response in cell survival is well communicated, HSP70, the mitochondrial Sirt3, and FoxO3a are relatively newly-determined players implicated in adaptive mechanisms [43-48]. Thus, it has recently been observed that HSP70 and Sirt3 can sustain cell radioresistance and antioxidant capacity of mitochondria respectively; and that FoxO3a can promote cell survival by inducing the expression of antioxidant enzymes, autophagy, and factors involved in cell cycle withdrawal, such as the cyclin-dependent kinase inhibitor (CKI) p27 [33, 44-48].

Although the transcription factors NFκB and FoxO3a are normally sequestered in the cytoplasm, ionizing irradiation, bacterial products, pro-inflammatory effectors, and oxidative stress can stimulate their nuclear translocation and DNA-binding activity [13, 42, 43]. NFκB and FoxO3a are known to regulate numerous genes, including autophagy genes, and therefore, could link responses to IR and bacterial challenge with up-regulation of autolysosomal activity [13, 40, 42, 43, 45]. We do not, however, exclude implication of stress-induced adaptors and chaperones such as the heat-shock proteins (HSPs). HSP70, in particular, was shown to promote cell radioresistance and can regulate autophagy [33, 46]. Therefore, we assumed that a battery of stress-sensitive mechanisms mediated by survival factors such as NFκB, FoxO3a, Sirt3, and HSP70 are involved in an adaptive response of MSCs to IR and bacterial challenge.

Immunoblotting analysis of stress-response proteins presented in Fig. 2A indicates that control MSCs had relatively high amounts of constitutively expressed HSP70 and (p65)NFκB and a detectable amount of Sirt3. These basal levels did not significantly change at 24 h following 8-Gy irradiation. A slight increase in HSP70, (p65)NFκB, and Sirt3 occurred only after 12-Gy irradiation. Up-regulation of Casp-3 was not detected in the irradiated MSCs (Fig. 2A), which suggested the absence of pro-apoptotic alterations. Additional bacterial challenge of the 8-Gy irradiated MSCs did not compromise their viability (Fig. 1D) and did not affect the profile of the stress-proteins, except that IR induced a significant increase in Sirt3, a mitochondrial stress-response protein, and MMP3, the type 3 matrix metalloproteinase, essential for remodeling of extracellular matrix (Fig. 2B).

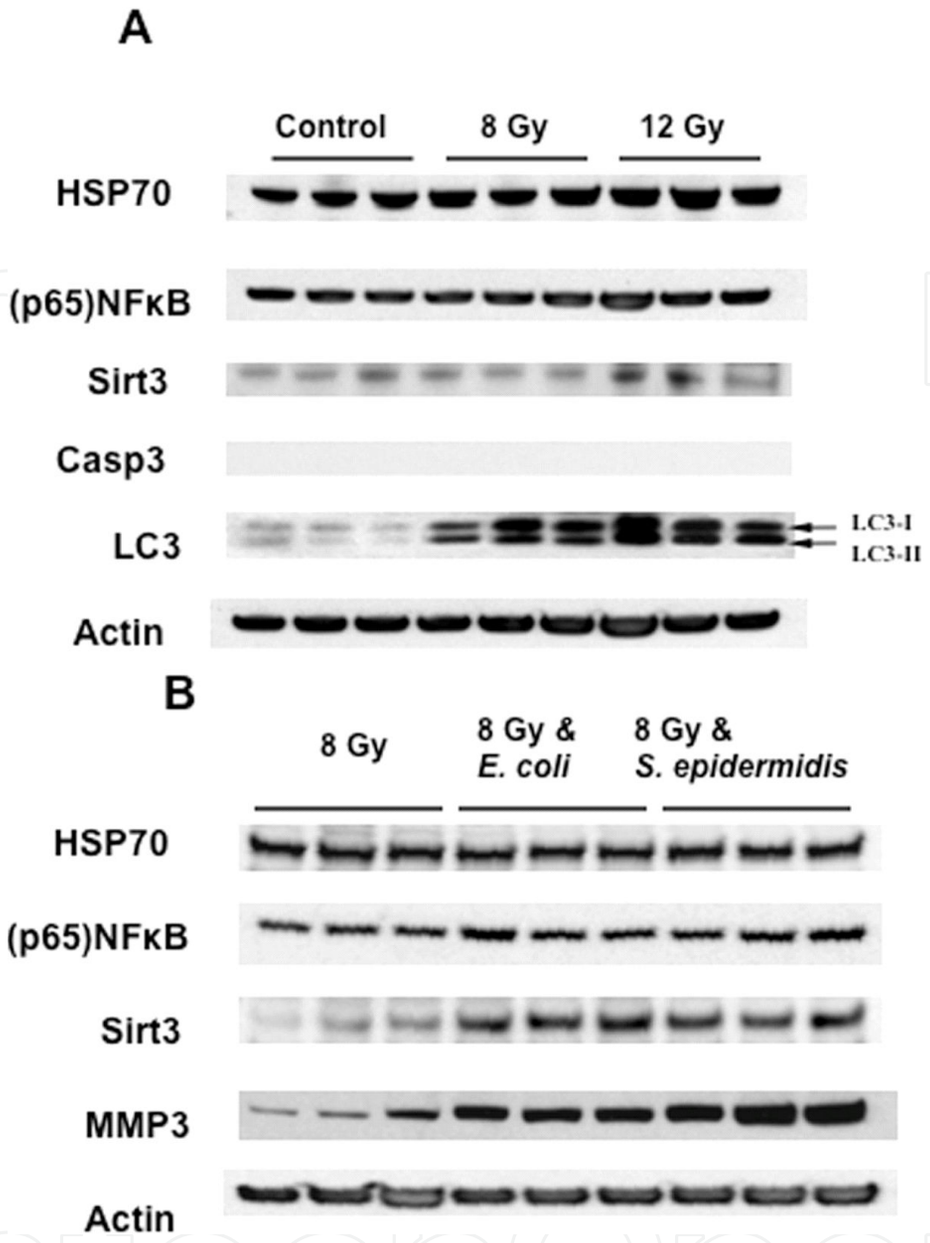


Figure 2. Immunoblot analysis of stress-response proteins in MSCs subjected to irradiation and bacterial challenge. A. MSCs, control and irradiated with 8 Gy and 12 Gy. Conditions: MSCs were harvested 24 h after irradiation then lysed and subjected to immunoblot analysis for stress-response proteins (HSP70, NFκB, and Sirt3), autolysosomal proteins (LC3-1 and LC3-II), and pro-apoptotic protein Caspase-3. B. MSCs irradiated with 8 Gy were challenged with either *E. coli* or *S. epidermidis*. Conditions: Irradiated MSCs were challenged with approximately $5x10^7$ bacteria /ml for 3 h in MesenCult Medium (without antibiotics). The cells were harvested and lysed 24 h after challenge. The protein lysates were subjected to immunoblot analysis for stress-response proteins (HSP70, NFκB, Sirt3, and MMP3).

Although in these experiments we did not observe a significant alteration of the amount of (p65)NFκB (Fig. 2B), the response of NFκB to IR was characterized by re-compartmentalization of (p65)NFκB resulting in an increase in its nuclear fraction (Fig. 3A). It should be noted that pre-incubation of the cells with pyrrolidine dithiocarbamate, an inhibitor of NFκB translocation, or wortmannin, an inhibitor of autophagy, resulted in development of pro-apoptotic

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alterations and loss of confluency after irradiation (not shown). Immunofluorescence imaging of spacial localization of FoxO3a in MSCs (Fig. 3B) indicated that FoxO3a response to IR was associated with an increase in its nuclear fraction in a manner similar to (p65)NFκB.

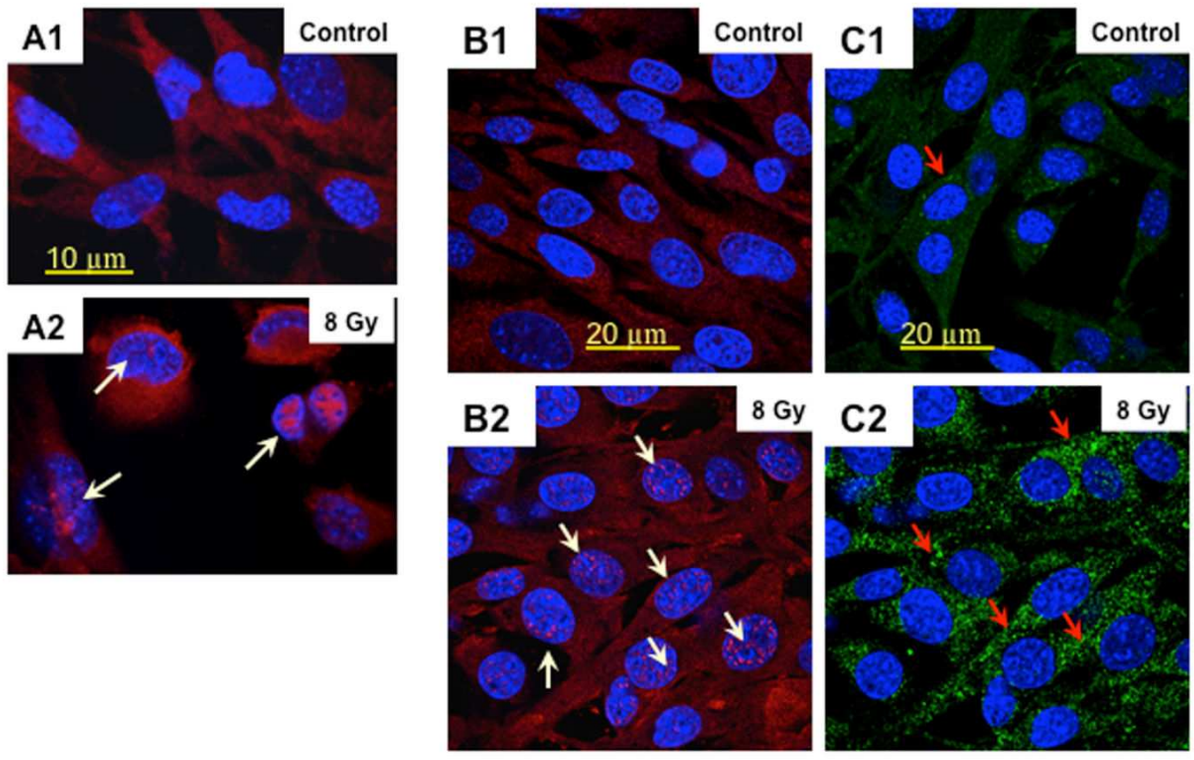


Figure 3. Confocal immunofluorescence imaging of nuclear translocation of NFκB and FoxO3a and activation of autophagy in MSCs subjected to 8-Gy irradiation. A. Projections of NFκB (red) in MSCs: A1, control; A2, 24 h after irradiation. Increase of nuclear fraction of p65 subunit of NFκB was observed in the irradiated cells due to transactivation of NFκB (indicated with white arrows). B. Projections of FoxO3a (red) in MSCs: B1, control; B2, 24 h after irradiation. Increase of nuclear fraction of FoxO3a was observed in the irradiated cells due to transactivation of FoxO3a (indicated with white arrows). C. Projections of LC3-positive autophagy vacuoles (green) in MSCs: C1, control; C2, 24 h after irradiation. A massive accumulation of autophagosomes occurred in irradiated MSCs (indicated with red arrows). Counterstaining of nuclei was with Hoechst 33342 (blue channel). The confocal images were taken with pinhole setup to obtain 0.5 µm Z-sections.

3.2. Autophagy—Autolysosomal response and secretory-activity in the irradiated MSCs subjected to bacterial challenge

The autophagy-autolysosomal pathway is considered to be an evolutionarily developed pro-survival mechanism, the purpose of which is to remove damaged and misfolded proteins, compromised organelles, and pathogens including bacteria [12, 21, 29, 34, 40]. A key step in autophagosome biogenesis is the conversion of light-chain protein 3 type I (LC3-I, also known as ubiquitin-like protein, Atg8) to type II (LC3-II). The conversion

occurs via the cleavage of the LC3-I carboxyl terminus by a redox-sensitive Atg4 cysteine protease. The subsequent binding of the modified LC3-I to phosphatidylethanolamine, i.e., process of lipidation of LC3-1, on the isolation membrane, as it forms, is mediated by E-1- and E-2-like enzymes Atg7 and Atg3 [22, 26, 27, 40, 49]. Therefore, conversion of LC3-I to LC3-II and formation of LC3-positive vesicles are considered to be a marker of activation of autophagy [22, 26, 27, 33, 40]. Notably, a growing body of reports suggests implication of FoxO3a and HSP-70 in regulation of LC3 expression and translocation [32, 43, 45].

A line of evidence suggests that autophagy is a more selective process than the "bulk process" as it was originally defined [40, 49]. The discovery and characterization of autophagic adapters like p62/sequestrosome 1 (SQSTM1) and NBR1 (neighbor of BRCA1 gene 1), and target-ubiquitination with small ubiquitin-like modifier 1 (SUMO1) has provided mechanistic insight into this process. p62/SQSTM1 and NBR1 are both selectively degraded by autophagy and are able to act as cargo receptors for degradation of ubiquitinated/sumoylated substrates. A direct interaction between these autophagic adapters and the autophagosomal marker protein LC3-II, mediated by a so-called LIR (LC3-interacting region) motif, and their inherent ability to polymerize or aggregate, as well as their ability to specifically recognize substrates, are required for efficient selective autophagy [40, 49].

We hypothesized that autophagy and xenophagy (i.e., selective degradation of foreign pathogens by autophagy) can be implicated in the pro-survival response of MSCs to IR-related damage and bacterial challenge. To address this hypothesis we conducted immunoblotting and immunofluorescence confocal imaging of autophagy MAP (LC3) protein, lysosomal LAMP1 and SUMO1 in MSCs after irradiation and challenge with *E. coli* and *S. epidermidis*.

The immunoblotting analysis of MSC proteins revealed a drastic increase in LC3-I and LC3-II (compared to control) at 24 h following 8-Gy and 12-Gy irradiation (Fig. 2A). These results suggested that, indeed, the autophagy MAP (LC3) pathway is implicated in that MSC response to IR-induced injury. In contrast to MAP (LC3), we did not observe a substantial increase in HSP-70. This was most likely due to relatively high background expression of this stress-response protein in the cells (Fig. 2A).

The above immunoblotting results were corroborated by the immunofluorescence confocal image analysis of the LC3 protein in MSCs. Thus, the data presented in Fig. 3C suggest that up-regulation of LC3-I/LC3-II proteins in the 8-Gy-irradiated cells was associated with massive formation of the LC3-positive vesicles which are well-documented to be features of autophagy [12, 13, 33]. The further TEM-assessment of the 8-Gy irradiated MSCs (in comparison with controls) revealed the presence of multiple vacuoles, which were formed by double-layer membrane and sequestered constituents of different densities (Figs. 4 A-C). Some of these vacuoles can be identified as secretory autolysosomes by the presence of multilamellar structures (most likely fibers of collagen) released extracellularly (Figs. 4 D and E), while others contained fractured organelles including mitochondria (Figs. 4 C and F).

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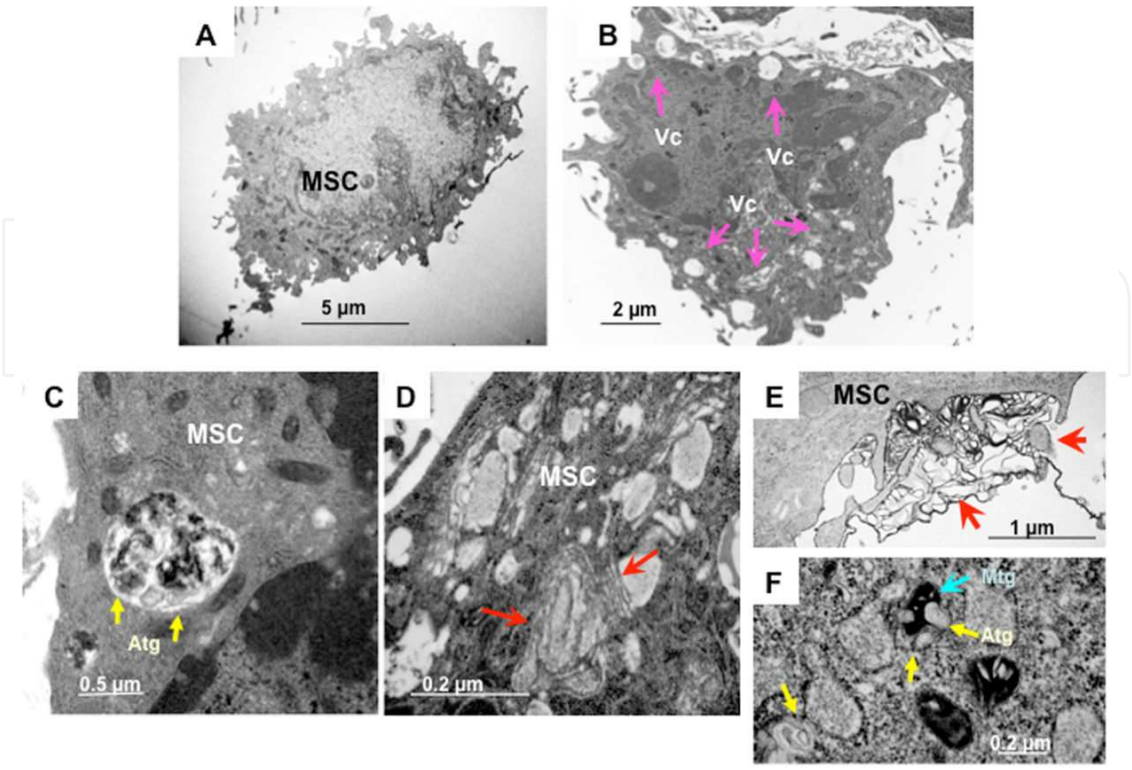


Figure 4. Transmission electron (TEM) analysis of autolysosomal vacuoles in irradiated MSCs. Panel A: A control MSC. Panel B: Irradiated MSC. A massive formation of different density autolysosomal vacuoles occurred after irradiation (indicated with pink arrows). Specimens were fixed 24 h after irradiation with 8 Gy gamma-photons. Panel C: Autolysosome sequestering cellular constituents (indicated with yellow arrows) in an irradiated MSC. Panel D: Formation of secretory autolysosomes containing multilamellar structures (indicated with red arrows) in an irradiated MSC. Panel E: Extracellular secretion of multilamellar structures from an irradiated cell. Panel F: Autolysosome sequestering mitochondria, *e.g.,* mitophagy, (indicated with blue arrow) in an irradiated MSC. Abbreviations: "Vc", vacuoles; "Atg", autophagosomes/autolysosomes; "Mtg", mitophagy.

In our recent research we demonstrated that intact MSCs are able to up-regulate autophagy in response to challenge with *E. coli* and employ this mechanism for inactivation of the microorganisms [13]. The data presented in this report showed that the irradiated MSCs retained their ability to phagocytise bacteria in a manner similar to that of non-irradiated MSCs (Fig. 5). Indeed, sequestration and degradation of *E. coli* and *S. epidermidis* in the MSC vacuoles, constituted by characteristic autophagosomal membranes, was observed at 5 h after bacterial challenge of both non-irradiated and irradiated MSCs (Figs. 5 A, B, D, and E).

The immunofluorescence confocal image analysis of the irradiated MSCs challenged with bacteria showed that the vacuoles containing bacteria were LC3-positive and that this LC3 immunoreactivity was co-localized with immunoreactivity to LAMP1, a marker of lysosomes, indicating presence of fusion of autophagosomes with lysosomes, i.e., formation of autolysosomes (Fig. 6). This increase in autolysosomal activity was accompanied by accumulation of the proteins LC3-II, a marker of up-regulation of autophagy, LAMP1, and p62/SQSTM1, a target adaptor (Figs. 7A and C). Meanwhile, the level of SUMO1, a target modifier protein, in the cells decreased after bacterial challenge (Fig. 7A). Interestingly, irradiation and bacterial

challenge resulted in up-regulation of the factors responsible for modification of extracellular matrix, such as collagen III, MMP3, and MMP13, i.e., the collagenase-3, (Fig. 7C), indicating that the stress-response aimed at multiple targets including extracellular ones.

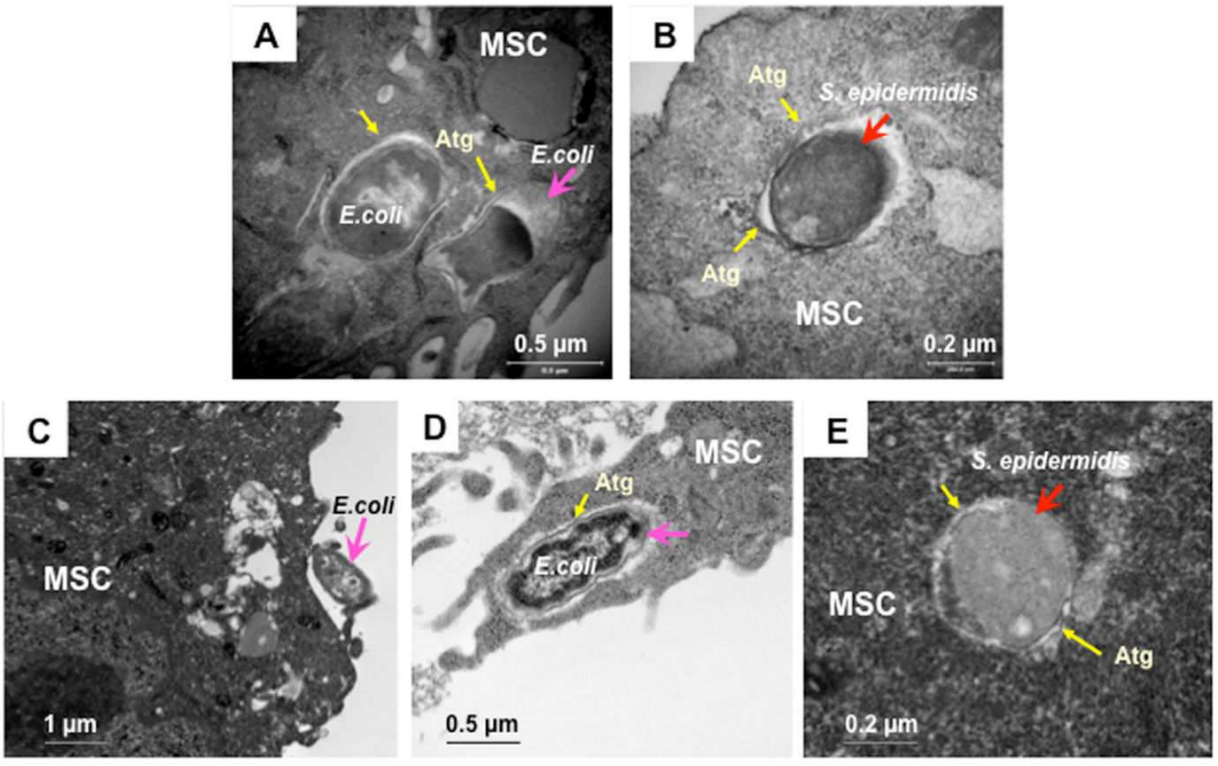


Figure 5. Assessment of phagocytosis and autophagy/autolysosomal processing of *E. coli* or *S. epidermidis* in irradiated MSCs with transmission electron microscopy. Panels A and B: Autolysosomal degradation of phagocytized *E. coli* and *S. epidermidis* in control MSCs. Autophagosome (ATG) membranes are indicated with yellow arrows. Conditions: Control MSCs were challenged with ~5x10^7 bacteria /ml for 3 h as indicated in *Methods*. The cells were harvested and fixed for TEM 5 h after challenge. Panels C, D, and E: TEM micrographs obtained from the 8-Gy irradiated cells. C - Engulfing and up-take of *E. coli* (pink arrows) by the cell plasma membrane extrusions (black arrows). D - Autolysosomal degradation of phagocytized *E. coli.* E - Autolysosomal degradation of phagocytized *S. epidermidis.* Atg, autophagosomes/autolysosomes. Conditions: 8-Gy irradiated MSCs were challenged with ~5x10^7 bacteria/ml for 3 h as indicated in *Methods*. The cells were harvested and fixed for TEM 5 h after challenge.

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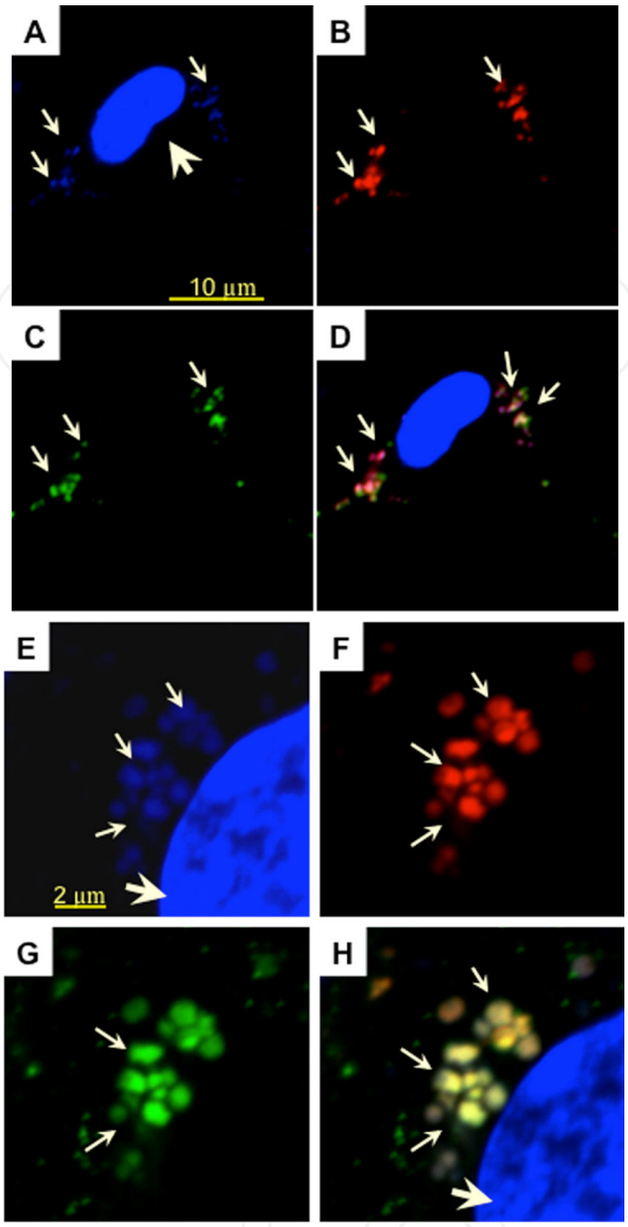


Figure 6. Confocal immunofluorescence imaging of autolysosomal sequestration of *E. coli* and *S. epidermidis* microorganisms phagocytized by MSCs irradiated at 8-Gy. Panel A – (Blue channel). *E. coli* (small arrows) and MSC nuclear DNA (large arrow) are indicated. Panel B – (Red channel). Spatial localization of LAMP1 is indicated with arrows. Panel C – (Green channel). Spatial localization of LC3 is indicated with arrows. Panel D – Overlay of images appeared in the blue, red, and green channels and presented in panels A, B, and C, respectively. Spatial co-localization of LAMP1, LC3, and *E. coli* DNA is indicated with arrows. Conditions: 8-Gy irradiated MSCs were challenged with ~5x10^7 *E. coli*/ml for 3 h. The cells were fixed 24 h after challenge. The fixed cells were subjected to immunofluorescence analysis for autolysosomal proteins. Panel E – (Blue channel). *S. epidermidis* (small arrows) and MSC nuclear DNA (large arrow) are indicated. Panel F – (Red channel). Spatial localization of LAMP1 is indicated with arrows. Panel G – (Green channel). Spatial localization of LC3 is indicated with arrows. Panel H – Overlay of images appeared in the blue, red, and green channels and presented in panels E, F, and G, respectively. Spatial co-localization of LAMP1, LC3, and *S. epidermidis* DNA is indicated with arrows. Conditions: 8-Gy irradiated MSCs were challenged with ~5x10^7 *S. epidermidis*/ml for 3 h. The cells were fixed 24 h after challenge. The fixed cells were subjected to immunofluorescence analysis for autolysosomal proteins. Counterstaining of nuclei was with Hoechst 33342 (blue channel). The confocal images were taken with pinhole setup to obtain 0.5 µm Z-sections.

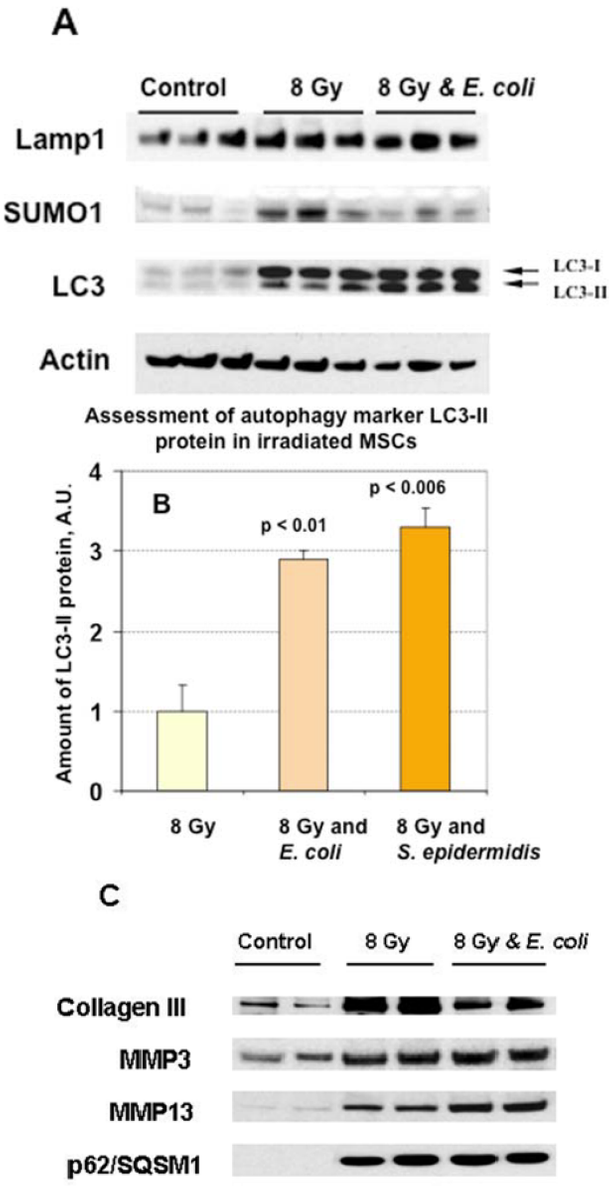


Figure 7. Immunoblot assessment of autolysosomal response in MSCs subjected to 8-Gy irradiation followed by challenge with either *E. coli* or *S. epidermidis.* Panel A. Representative immunoblotting bands of SUMO1, LC3, and LAMP1. Note that irradiated MSCs were challenged with *E. coli.* Panel B. Densitometry histograms of LC3-II bands of the immunoblots of proteins from the irradiated MSCs subjected to challenge with *E. coli* or *S. epidermidis*. The presented bars indicate the relative density of LC3-II protein (normalized to density of actin bands). The statistical significance was determined by Student's *t*-test (n=3). Panel C. Representative immunoblots of collagen III, MMP3, MMP13, and p62/SQSM1. Note that irradiated MSCs were challenged with *E. coli.* Conditions: Irradiated MSCs were challenged with approximately $5x10^7$ bacteria/ml for 3 h as indicated in *Methods*. The cells were harvested and lysed 24 h after challenge.

Various cells eliminate bacterial microorganisms by autophagy, and this elimination is in many cases crucial for host resistance to bacterial translocation. Targeting of microorganisms can occur outside of the host cells in extracellular matrix by different defense mechanisms, such as the cell-produced oxidative burst, nitric oxide, antibacterial peptides, and extracellular traps [50]. The data presented in the present report (Figs. 2, 4, 5, 7) suggest that MSCs can employ

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the autophagy mechanism to modify extracellular matrix by releasing collagen and matrix metalloproteases in order to increase efficacy of extracellular entrapment, uptake, and further phagocytosis of the microorganisms.

Recent observations suggest that autophagosomes do not form randomly in the cytoplasm, but rather sequester the bacteria selectively [23, 49, 51]. Therefore, autophagosomes that engulf microbes are sometimes much larger than those formed during degradation of cellular organelles, suggesting that the elongation step of the autophagosome membrane is involved in bacteria-surrounding autophagy [13, 32]. This effect could be observed by comparison of profiles of the autophagosomes, which appeared in the irradiated MSCs before and after challenge with bacteria (Figs. 3 and 6). The mechanism underlying selective induction of autophagy at the site of microbe phagocytosis remains unknown. However, it is likely mediated by pattern recognition receptors, stress-response elements, adaptor proteins, e.g., p62/SQSTM1, and ubiquitin-like modifiers, which can target bacteria and ultimately recruit factors essential for the formation of autophagosomes [21, 22, 52].

4. Conclusion

Survival of multicellular organisms in a non-sterile environment requires a network of host defense mechanisms. The initial contact of pathogenic and opportunistic microorganisms with a host usually takes place at internal or external body surfaces. Microbial growth and translocation are controlled by multi-layer integrative tissue barriers that mediate innate defense mechanisms. Tissue injury compromises barrier function, and increases risk of infection and sepsis. Recent observations from our laboratory indicate that ubiquitous MSCs can modulate systemic responses to bacterial infection and support tissue repair and healing when recruited at sites of injury [2, 5, 9, 14-18]. However, these "compensatory" responses of MSCs can be skewed and suppressed after irradiation. To elucidate the role of autophagy in response of stromal cells to radiation injury and bacterial infection we irradiated cultured MSCs and challenged them with *S. epidermidis* or *E. coli.* Using this cell model we showed that (i) irradiation induced translocation of cytosol NF-kB and FoxO3a to the nucleus; (ii) irradiation and bacterial challenge induced increases in Sirt3 stress-response factors, LC3, MMP3, MMP13, collagen III, SUMO1, and p62/SQSM1 proteins; and (iii) the antibacterial defense response of the irradiated MSCs was characterized by extensive phagocytosis and inactivation of both *S. epidermidis* or *E. coli* in autolysosomes.

Our communication is the first report demonstrating a potential role of MSCs in sustaining antibacterial barrier functions of irradiated tissues. We postulate that effector mechanisms expressed by MSCs can contribute to the innate defense response to IR injury alone or, especially, when IR is combined with trauma.

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Disclaimer

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