Data independent acquisition mass spectrometry of irradiated mouse lung endothelial cells reveals a STAT-associated inflammatory response

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Key words: cGAS/STING pathway, endothelial cell, ionizing radiation, pulmonary inflammation, STAT1, DIA

Total word count: 5,204

**Abbreviations**

AGC Automatic gain control

BCA Bicinchoninic acid

CBCT Cone beam computed tomography

CD31 Cluster of differentiation 31

cGAMP Cyclic guanosine monophosphate-adenosine monophosphate

cGAS Cyclic GMP-AMP synthase

DDA Data-dependent acquisition

DIA Data-independent acquisition

dsDNA Double stranded deoxyribonucleic acid

ECL Enhanced chemoluminescence

EGM2 Endothelial cell growth medium

FASP Filter-aided sample preparation

GBP2 Guanylate-binding protein 2

Gy Gray

HCD High collisional dissociation

HCECest2 Human coronary artery endothelial cell line

HRM Hyper reaction monitoring

HUVEC Human umbilical cord endothelial cell

IFI10 10 kDa interferon gamma-induced protein

IFI30 Gamma-interferon-inducible lysosomal thiol reductase

IFI44 Interferon-induced protein 44-like

IFN Interferon

IL-1 Interleukin-1 alpha

IL-6 Interleukin-6

IL-10 Interleukin 10

IPA Ingenuity Pathway Analysis

IRF2BP2 Interferon regulatory factor 2-binding protein 2

iRT Indexed retention time

ISG15 Ubiquitin like protein interferon stimulated protein 15 kDa

JAK Janus kinase

MAPK Mitogen-activated protein kinase 1

MNDA Myeloid cell nuclear differentiation antigen

p16 Cyclin dependent kinase-inhibitor 2A

p21 Cyclin dependent kinase-inhibitor 1

PRXD5 Peroxiredoxin 5

RIPA Radioimmunoprecipitation assay buffer

RSLC Rapid separation liquid chromatography

SASP Senescence-associated secretory phenotype

SARRP Small Animal Radiation Research Platform

SOD1 Superoxide dismutase [Cu-Zn]

STAT1 Signal transducer and activator of transcription 1

STAT3 Signal-transducer and activator of transcription 3

STING Stimulator of interferon genes protein

TNFα Tumor necrosis factor alpha

**Abstract**

Pulmonary inflammation is an adverse consequence of radiation therapy in breast cancer. The aim of this study was to elucidate biological pathways leading to this pathology. Lung endothelial cells were isolated 24h after thorax-irradiation (sham or 10 Gy x-ray) of C57Bl/6 mice and cultivated for 6 days. Quantitative proteomic analysis of lung endothelial cells was done using data independent acquisition (DIA) mass spectrometry. The data were analyzed using Ingenuity Pathway Analysis and STRINGdb. In total, 4,220 proteins were identified using DIA of which 60 were dysregulated in the irradiated samples (fold change + 2.0, q-value < 0.05). Several upregulated proteins (12/40) formed a cluster of inflammatory proteins with STAT1 and IRF3 as predicted upstream regulators. The several-fold increased expression of STAT1 and STAT-associated ISG15 was confirmed by immunoblotting. The antioxidant proteins SOD1 and PRXD5 were found to be downregulated in the immunoblot assay suggesting enhanced oxidative stress in the irradiated tissue. Similarly, the phosphorylated (active) forms of STING and IRF3, both members of the cGAS/STING pathway, were downregulated. These data suggest the involvement of JAK/STAT and cGas/STING pathways in the genesis of radiation-induced lung inflammation. These pathways may be of use as targets for prevention of radiation-induced lung damage.

1. Introduction

Breast cancer is the most common diagnosed cancer in women [1]. Radiation therapy is an effective and often used treatment [2], whereby lung tissue unavoidably gets partially irradiated [3]. This results frequently in pulmonary inflammation possibly due to local release of cytokines and other pro-inflammatory molecules [4]. The acute inflammatory response is chiefly orchestrated by endothelial cells of the pulmonary vasculature [5]. The early induction of inflammation may cause persistent low-level inflammation that is characterized by endothelial dysfunction and senescence [6-8], finally leading to pneumonitis and fibrosis [4].

We previously used a human coronary artery endothelial cell line to study the biological mechanism of radiation-associated inflammation [9]. The endothelial cells entering radiation-induced premature senescence showed increased expression of STAT-proteins. The JAK/STAT-pathway is prominent in the adaptive and innate immune response [10]. One of the key-players in the STAT cascade is STAT1, a transcription factor that induces expression of interferon (IFN)-related and other inflammatory genes [11].

A potent inducer of STAT1 and other type I IFN-proteins is the so-called cGAS/STING-pathway, a component of the innate immune system that alerts the cell´s immune system after detecting the presence of cytosolic DNA [12, 13]. In a convoluted cascade, the protein cGAS, activated by dsDNA, produces the second messenger cGAMP that binds and activates the adaptor protein STING that, in its turn, activates transcription factor IRF3 in a phosphorylation-dependent mechanism [14]. This triggers the transcription of pro-inflammatory genes [15]. Radiation-induced activation of the cGAS/STING-pathway followed by increased expression of STAT1 has been shown in different cancers [16].

An important player in converting acute inflammation into a chronic one is ISG15, a small secretory protein, the expression of which is induced by the JAK/STAT pathway [17, 18]. It covalently modifies cytoplasmic and nuclear proteins by ISGylation, similar to ubiquitination [19]. In a feedback loop, ISG15 is able to induce cytokine expression and augment inflammation by modulating the JAK/STAT proteins and stabilizing STAT1 [18, 20].

The aim of this study was to investigate the mechanism of the radiation-induced inflammatory response in lung endothelium. For this purpose, we used label-free proteomics based on data-independent acquisition. Since irradiation creates DNA double strand breaks, our particular interest was to study the role of cGAS/STING pathway downstream of damaged DNA. Using a mouse model, primary lung endothelial cells were isolated 24 h post-irradiation (10 Gy) and further cultured for six days. A significant induction of STAT1 and ISG15 was seen, whilst the cGAS/STING-pathway showed inactivation.

1. Material and Methods

**2.1 Animals and irradiation**

All experiments were approved by the responsible state agency (Regierung von Oberbayern, certificate no. 55.2-1-54-2532- 191-14). They were in compliance with national law on animal experimentation and welfare and performed in accordance with institutional and ARRIVE guidelines. Female C57Bl/6 mice (Charles River Laboratories, Sulzfeld, Germany) aged 4-5 weeks were randomly allocated to the treatment groups (4 mice/group). Irradiation of the thorax was performed using a high-precision image-guided Small Animal Radiation Research Platform (SARRP, Xstrahl, Camberley, UK). The mice were anesthetized by isoflurane/oxygen inhalation for the duration of the treatment. The thorax was first visualized by CBCT to identify the required radiation field (60-kV and 0.8-mA photons filtered with aluminum 1 mm). The software SARRP control and Muriplan were used to precisely target thorax and estimate the irradiation dose, respectively. A single dose of 10 Gy was delivered using a 220 kV and 13 mA X-ray beam filtered with copper (0.15 mm). Control mice received sham irradiation using CBCT. The mice were housed in single ventilated cages under pathogen-free conditions while experimentation.

**2.2 Primary cell culture**

Primary microvascular CD31-positive endothelial cells were isolated from the lung as described in detail previously [21]. For each biological replicate, endothelial cells of four mice were pooled and seeded in gelatin (2%; Merck KGaA, Darmstadt, Germany)-coated ibidi® chamber (ibidi GmbH, Gräfelfing, Germany) with EGM2 (PromoCell, Heidelberg, Germany) supplemented with 10% FCS, streptomycin (100 mg/ml) and penicillin (100 U/ml). The cells were cultured for six days with daily medium change.

**2.3 Protein lysis and determination of protein concentration**

RIPA buffer (Pierce Biotechnology, Rockford, IL, USA) was used for cell lysis. Protease inhibitor (cOmplete tablets) and phosphatase inhibitor (PhosSTOP, both Roche Diagnostics GmbH, Mannheim, Germany) were added prior to lysis. RIPA buffer was applied into slides that were shortly frozen (10 min, -20 °C) to remove the adherent cells. The cells were washed out with RIPA buffer and the lysate was sonicated and centrifuged for 20 min at 4 °C with 13,000 g. Supernatants were collected and stored until further use at -80 °C. Protein concentration was measured by BCA protein assay (Pierce Biotechnology) according to manufacturer’s instructions. The analysis was performed at 562 nm on an Infinite M200 (Tecan GmbH, Crailsheim, Germany).

**2.4 Sample preparation**

Ten µg of protein lysate was subjected to tryptic digestion using a modified FASP protocol (Wisniewski et al., 2009; Grosche et al., 2016).

**2.5 Mass spectrometry (MS) measurement**

MS data were acquired in data-independent acquisition (DIA) mode on a Q Exactive (QE) high field (HF) mass spectrometer (Thermo Fisher Scientific GmbH, Bremen, Germany). Samples were automatically loaded to the online coupled RSLC (Ultimate 3000, Thermo Fisher Scientific GmbH) HPLC system. A nano trap column was used (300 μm inner diameter × 5 mm, packed with Acclaim PepMap100 C18, 5 μm, 100 Å; LC Packings, Sunnyvale, CA) before separation by reversed phase chromatography (Acquity UPLC M-Class HSS T3 Column 75 µm ID x 250 mm, 1.8 µm; Waters, Eschborn, Germany) at 40 °C. Peptides were eluted from the column at 250 nl/min using an acetonitrile (ACN) gradient (in 0.1 % formic acid) from 3 % to 40 % over 45 min.

The HRM DIA method consisted of a survey scan from 300 to 1500 *m/z* at 120 000 resolution and AGC target of 3\*106 or 120 ms maximum injection time. Fragmentation was performed *via* HCD with a target value of 3\*106 ions determined with predictive AGC. Precursor peptides were isolated with 17 variable windows spanning from 300 to 1500 *m/z* at 30 000 resolution with an AGC target of 3\*106 and automatic injection time. The normalized collision energy was 28 and the spectra were recorded in profile type.

**2.6 Spectral library**

The spectral library was generated as described previously [22]. The final spectral library generated in Spectronaut contained 11,184 protein groups and 349,634 peptide precursors.

**2.7 Spectronaut analysis and data processing**

The DIA MS data for lung endothelial cell groups were analyzed using the Spectronaut 10 software applying default settings with the exception: quantification was limited to proteotypic peptides, data filtering was set to q-value 50 % percentile, summing-up peptide abundances.

**2.8 Immunoblotting**

Five microgram of proteins lysate were loaded on 1D Bio-Rad Mini-Protean® TGX Stain-FreeTM 4-15 % Gels (Bio-Rad Laboratories GmbH, Munich, Germany). Proteins were denatured in 1 x Laemmli buffer for 5 min at 95 °C. Gel runs were performed for 2 h at 60 to 120 V. Blotting was performed using a Bio-Rad Trans-Blot® TurboTM Semi-dry transfer system. A predefined program of 1.3 A for 10 min was used to transfer also high-molecular weight proteins onto 0.2 µm Nitrocellulose membrane (Bio-Rad Laboratories GmbH). For visualization of protein separation on the gel, transfer efficiency the internal control Stain-freeTM technology after one minute of activation time was used. Membranes were washed and blocked with 8 % milk (milk powder, Carl Roth GmbH & Co KG, Karlsruhe, Germany, dissolved in 1 x TBST) for 1 h. Antibodies were used according to manufacturer’s instructions and diluted in 5 % milk if not further specified. Following antibodies were purchased from Cell Signaling (Cell Signaling Technology Europe B.V., Frankfurt am Main, Germany): phospho STING (85735), phospho IRF3 (S396, 4947), cGAS (15102), phospho p38 MAPK (T180/Y182), (9211), p38 MAPK (9212), from Abcam (Abcam plc, Cambridge, UK): peroxiredoxin 5 (ab119712), from R&D Systems (R&D Systems, Inc., Abingdon, UK): STING (MAB7169) and from Santa Cruz (Santa Cruz Biotechnology, Inc., Heidelberg, Germany): SOD1 (sc-11407), STAT1 (sc-346), ISG15 (sc-166755).

Membranes were incubated with the primary antibody overnight at 4 °C. For detection, the blots were incubated for 2 h with the appropriate horseradish-peroxidase conjugated anti-rabbit or -mouse secondary antibody. Detection was performed by ECL Advance Western blotting detection kit (GE Healthcare GmbH, Solingen, Germany) quantified in a chemiluminescence reader ChemiDocTM MP (Bio-Rad Laboratories GmbH) with the appropriate ImageLab 6.0.1 software. Blots were stripped for reprobing maximally twice with Stripping Buffer (0.2 M Glycin, 0.003 M SDS, 1/100 Tween20, pH 2.4).

Protein band intensities obtained by chemiluminescence visualization were normalized by total protein staining based on StainfreeTM technology. The corresponding blots are shown in Supplementary Figure S1.

**2.9 Bioinformatics analysis**

To evaluate protein expression in the normalized dataset one-way ANOVA approach by limma package in R (https://www.R-project.org/) was used. Western blotting band intensity was analyzed by Student’s t-test. IPA (Qiagen GmbH, Hilden, Germany) and STRING [23] (medium confidence, 0.400) were used to cluster and classify pathways.

**2.10 Statistical analysis**

Filtering criteria for proteomics analyses were the following: significance for fold change (ratio irradiated to sham-irradiated) ≥2.00 or ≤0.50 and a FDR (q) ≤ 0.05 (Benjamini-Hochberg). Immunoblotting significance criteria: Proteins showing altered expression compared to the control were considered to be significant if p ≤ 0.05 (unpaired Student’s t test). The error bars were calculated as standard deviation.

All experiments were performed using at least three biological replicates. The principal component analysis (PCA) based on all proteomic features showed one outlier in the control group that was excluded from further experiments (Supplementary Figure S2).

**2.11 Data availability**

Raw files of MS runs are available under the following link: <http://dx.doi.org/doi:10.20348/STOREDB/1152/1212>.

1. Results

**3.1 Proteomics analysis**

In total, 4,220 proteins were identified with at least one peptide using Spectronaut (Supplementary Table S1). Of those, 4,208 proteins could be quantified by a fold change and p-value (< 0.05) (Supplementary Table S2). Using Benjamini-Hochberg corrected p-values (q-values) 589 proteins qualified for quantification (Supplementary Table S3). Of these, 60 were deregulated by a fold change of + 2.0 (Table 1) with 40 proteins showing upregulation and 20 downregulation as illustrated in Figure 1A. The corresponding Volcano plot is shown in Figure 1B.

A supervised hierarchical clustering (heat map) established the separation of the four irradiated samples from the three non-irradiated controls (Figure 2A). A scatter plot showing clear correlation within all samples in the two treatment groups, thereby supporting the used statistical tools, is shown in Supplementary Figure S3.

**3.2 Bioinformatics analysis**

Protein interactions of the 60 deregulated proteins were investigated using the STRING database. Two main clusters were seen: (i) a cluster consisting of pro-inflammatory proteins (light green) and (ii) a cluster representing proteins of ribosomal and histone origin (dark green) (Figure 2B). All proteins of the inflammatory cluster including STAT1, ISG15, and GBP2 were upregulated, whilst the members of the ribosome/histone cluster were downregulated (Table 1). The other deregulated proteins did not build clusters that could have been defined under any particular biological function (Figure 2B, shown in gray).

All deregulated proteins were uploaded into IPA for upstream regulator prediction. The predicted upstream regulators, based on the activation z-score of >2 (IPA), were STAT1 and IRF3 (Figure 2C).

**3.3 Immunoblotting validation**

Immunoblotting was performed to validate the proteomics findings. Firstly, the expression of inflammatory proteins STAT1, ISG15, p38, and STAT3 was quantified. In accordance with the proteomics data, the level of the STAT1 and ISG15 was significantly upregulated after irradiation (Figure 3A, 3B). The total amount of p38 or phospho-p38 (pp38) was not changed (Figure 3C, 3D). The ratio pp38/p38 showed tendency to upregulation that did not quite reach statistical significance (p = 0.0502) (Figure 3E). No changes were observed in the level of STAT3 (data not shown).

Since we previously observed a downregulation of oxidative stress markers in irradiated endothelial cells [9], oxidative stress response was investigated (Figure 3F-3H). The expression of antioxidative proteins SOD1 and PRXD5 (isoform 2) was downregulated after irradiation. This was probably due to radiation-induced increase in the reactive oxygen species levels.

To further investigate the radiation-induced enhancement of inflammatory markers, the key players of the cGAS/STING-pathway: STING, IRF3, and cGAS were tested (Figure 3I-3L). In agreement with the proteomics data, the expression of total STING was not altered (Table S1). In contrast, the level of phospho-STING (S366) was significantly reduced in the irradiated samples. Similarly, the level of phospho-IRF3 (S396) showed radiation-induced decrease. Total IRF3 was not detectable by immunoblotting but was found unchanged in the proteomics analysis (Table S2). The level of the cGAS protein was not significantly altered with irradiation.

The expression of the p21Cip1/Waf1 protein, the upregulation of which has been seen in radiation-induced senescence in heart endothelial cells [9] showed no alteration in the lung endothelial cells one week post irradiation (data not shown).

1. Discussion

Irradiation is known to cause long-term damage in endothelial cells by inducing local and systemic inflammation [6, 7, 24] but the mechanism is largely unknown. We have shown previously that human coronary artery endothelial cells (HCECest2) are already in an inflammatory state two weeks post 10 Gy (X-ray) dose *in vitro*. The inflammatory response was reflected by increased levels of ISG15, p38, STAT1, and others [9]. The results of this study, using primary endothelial cells from irradiated lung, are in line with these findings. Radiation-associated inflammation was seen as significantly increased expression of Type I and Type II IFN-related proteins, especially ISG15, GBP2, IFI30, MNDA (IFI211) and, in particular, STAT1 (Table 1).

The phosphorylation of STAT1 is a transient process, increasing quickly but then decreasing over a period of some hours [25]. In contrast, increased expression of non-phosphorylated STAT1, as found here, has been associated with prolonged expression of INF-induced immune regulatory genes, including STAT1 itself [25, 26]. The p38 MAPK, the activation of which showed a positive trend in the irradiated cells (p = 0.0502), plays a critical role in Type I IFN-dependent transcriptional regulation but does not affect the phosphorylation status of STAT1 [27]. A particular role in the maintenance of pro-inflammatory status has been given to ISG15 that was found to be strongly upregulated in this study. It is a secretory protein [28] that is able to induce INF-gamma and IL-10 secretion [29] and thereby spread inflammation among endothelial cells in a paracrine manner [9]. In addition, the anti-inflammatory protein IRF2BPP2, important cofactor for revascularization [30], was downregulated in irradiated endothelial cells (Table 1), further emphasizing the pro-inflammatory character of the proteomic response. Many of the IFN-related proteins found to be deregulated in this study have been shown to react in response to interferons but also to stay activated over time in fibroblasts [17].

One of the potential inducers of type I IFN-related inflammation is the cGAS/STING-pathway [13]. Significant increase of interferon gamma-induced proteins 10 (IFI10) and 44 (IFI44) was seen in the lung of wild type mice 24 h after total body irradiation with 4.25 Gy but not in cGAS k. o. or STING k. o. mice, emphasizing the role of this pathway in the early radiation-induced pulmonary inflammation [31]. While inflammation is an important process in the response to infection, uncontrolled and prolonged inflammation may have serious adverse consequences [13, 32]. A number of negative feedback pathways in the control the cGAS/STING pathway have been suggested [33] but information concerning the induction and maintenance of the cGAS/STING pathway in radiation response is scarce. This study shows a significant reduction of the phosphorylated, transcriptionally active forms of STING and IRF3 in the irradiated lung endothelial cells compared to controls seven days after the exposure, suggesting a possible negative feedback loop after initial induction of the cGAS/STING pathway. The bioinformatics analysis predicted activated IRF3 to be the transcriptional regulator of the changes observed in the irradiated proteome (Fig. 2C), also indicating initial induction of this pathway. Taken together, a radiation-associated induction of the cGAS/STING pathway seems to be transient, whilst that of the STAT1-related pathway is permanent. However, more studies are needed to corroborate this.

In line with previous data [6, 9, 34] we observed here a downregulation of antioxidative proteins (SOD1, PRDX5) in irradiated endothelial cells. Interestingly, a STAT-dependent positive feedback loop leading to a sustained interferon signature has been associated with increased ROS production in HUVECs [35] that could at least partly explain the depletion of oxidative stress response proteins. In general, inflammation is known to be coupled to chronic oxidative stress in radiation-induced normal tissue injury [36].

In conclusion, this study provides first evidence of the involvement type I IFN-response and STAT1 in particular and cGAS/STING pathways in radiation-induced inflammation of the murine lung, greatly resembling the radiation response of human heart endothelial cells [9]. The aforementioned pathways should be considered as future therapeutic targets for potential prevention and treatment of pulmonary inflammation during radiation therapy.

**Acknowledgments**

This research was supported by the Federal Ministry of Education and Research of Germany (BMBF) with funding numbers 02NUK038B, and 02NUK045C.

**Conflict of interest statement**

The authors have declared no conflict of interest.

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**Figure legends**

Figure 1. A) Bar charts showing the deregulated proteins in mouse lung endothelial cells seven days post-irradiation. B) Volcano plot displaying down- (blue) and up- (yellow) regulated proteins by –Log10 q-value and Log2 fold change.

Figure 2. A) A heat map showing the 60 deregulated proteins. Samples are distributed according to the average linkage hierarchical clustering (c=controls, T=irradiated samples). Z-scores are calculated by subtraction of the mean and dividing by the standard deviation for the deregulated proteins. B) Interaction analysis of all significantly deregulated proteins using the STRING-db. The light green cluster represents the inflammatory cluster, the dark green cluster consists of ribosomal and histone proteins. The significance of deregulation was based on the label-free proteomics analysis (fold change of ±2.0; q < 0.05; n = 3/4). C) Analysis of predicted upstream regulators using IPA. Graphical presentation of deregulated proteins with their upstream regulator STAT1 and IRF3 in the irradiated cells is shown (http://www.INGENUITY.com). The upregulated proteins are marked in red; the orange color of the STAT1 and IRF3 nodes indicates activation.

Figure 3. Immunoblot verification of protein changes in the irradiated cells. STAT1 (A), ISG15 (B), total p38 (C), phospho p38 (D); the ratio p38/phosho p38 (E), SOD1 (F), PRDX5 isoforms 1 (G) and 2 (H), STING (I), phospho STING (J), phospho IRF3 (K) and cGAS (L) are shown. The bars represent the relative expression after correction for background and normalization to stain free. The error bars are calculated as SD (t test; \*p<0.05, \*\*p<0.01, \*\*\*p<0.005; n = 3/4).