Journal of Radiation Research, Vol. 64, No. 2, 2023, pp. 304–316
https://doi.org/10.1093/jrr/rrac094
Advance Access Publication: 19 January 2023

Journal of Radiation Research

OXFORD

Bystander signals from low- and high-dose irradiated human primary fibroblasts and keratinocytes modulate the inflammatory response of peripheral blood mononuclear cells

Isabelle Testard, Elizabeth Garcia-Chartier, Amani Issa[†], Véronique Collin-Faure, Catherine Aude-Garcia and Serge M. Candéias*

University Grenoble Alpes, CEA, CNRS, IRIG-LCBM-UMR5249, 38054, Grenoble, France
*Corresponding author. Laboratoire de Chimie et Biologie des Métaux, UMR 5259 CEA-CNRS-UGA, 17 avenue des martyrs, 38054 Grenoble Cedex 9, France. Tel: +33(0)4 38 78 92 49; Fax: +33(0)4 38 78 91 21. Email: serge.candeias@cea.fr
[†]Present address: Amani Issa, Ingénierie Moléculaire et Physiopathologie Articulaire, UMR 7365 CNRS-University of Lorraine, Biopôle de l'Université de Lorraine, 54505 Vandoeuvre-lès-Nancy, France
(Received 26 April 2022; revised 1 August 2022; editorial decision 30 November 2022)

ABSTRACT

Irradiated cells can propagate signals to neighboring cells. Manifestations of these so-called bystander effects (BEs) are thought to be relatively more important after exposure to low- vs high-dose radiation and can be mediated via the release of secreted molecules, including inflammatory cytokines, from irradiated cells. Thus, BEs can potentially modify the inflammatory environment of irradiated cells. To determine whether these modifications could affect the functionality of bystander immune cells and their inflammatory response, we analyzed and compared the *in vitro* response of primary human fibroblasts and keratinocytes to low and high doses of radiation and assessed their ability to modulate the inflammatory activation of peripheral blood mononuclear cells (PBMCs). Only high-dose exposure resulted in either up- or down-regulation of selected inflammatory genes. In conditioned culture media transfer experiments, radiation-induced bystander signals elicited from irradiated fibroblasts and keratinocytes were found to modulate the transcription of inflammatory mediator genes in resting PBMCs, and after activation of PBMCs stimulated with lipopolysaccharide (LPS), a strong inflammatory agent. Radiation-induced BEs induced from skin cells can therefore act as a modifier of the inflammatory response of bystander immune cells and affect their functionality.

Keywords: bystander effects (BEs); inflammation; low-dose radiation; high-dose radiation; gene expression; cytokines

INTRODUCTION

DNA lesions and activation of DNA damage signaling pathways can readily be observed in cells after irradiation [1, 2]. More surprising, DNA lesions, or their consequences, can also occur in cells that have not been irradiated but are located in the vicinity of exposed cells [3]. This phenomenon has been termed non-targeted effects (NTEs) or bystander effects (BEs). BEs have been demonstrated in many different experimental systems. They can be transmitted by direct cell–cell contact or via factors released in culture medium [4–6] and propagated through recipient cells [7, 8] and in tissues [9], possibly by microvesicles/exosomes [10–13]. Bystander cells can be irradiated or not. BEs can therefore act as a modifier of the response to radiation of irradiated cells [14, 15].

As NTEs were first showed to replicate in non-exposed cells some of the effects induced by radiation in irradiated cells, most of the endpoints analyzed were associated by the induction of—or the response to—genotoxic stress in bystander cells [3, 8, 9, 15–20]. Several pathways have been involved in the transmission of these effects, including MAPK [15, 16], p53 [8], NF-kB [21], cytokine/cytokine receptor interactions [22, 23], and modulation of the redox status [17, 18, 22–24]. BEs involve a large number of different proteins in irradiated cells, conditioned medium or bystander cells [25–28]. Interestingly, BEs

© The Author(s) 2023. Published by Oxford University Press on behalf of The Japanese Radiation Research Society and Japanese Society for Radiation Oncology.
This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (https://creativecommons.org/licenses/by-nc/4.0/), which permits non-commercial re-use, distribution, and reproduction in any medium, provided the original work is properly cited. For commercial re-use, please contact journals.permissions@oup.com

have been suggested to saturate above a certain radiation threshold [3, 18, 29]; consequently, their contribution to the overall radiation-induced effects might be relatively more important after low-dose radiation exposure [4, 5]. However, supernatant transfer experiments did show that BEs can be elicited from cells exposed to high-dose radiation [15, 16, 30, 31].

Inflammation is a defense mechanism resulting in the mobilization of immune cells in response to infection or tissue damage. The sensing of pathogens or tissue damage involves some of the same receptors on immune cells, including toll-like receptors (TLRs). The so-called sterile inflammation is initiated by molecules released/produced by tissue damage, stressed cells or cells dying by non-apoptotic mechanisms, collectively termed danger signals or danger associated molecular patterns (DAMPs) [32, 33]. These molecules include ATP, chromatin/DNA, cytokines and intra-cellular molecules like HMGB1 or uric acid [33, 34]. Radiation-induced sterile inflammation can be seen as a category of bystander signalization whereby irradiated cells alert immune cells that they have been damaged. [13] *In vivo*, the sensing of extra-cellular danger signals by TLRs can modulate the activity of immune cells. For example, radiation exposure can lead to long lasting activation of inflammatory networks, including some usually activated after DAMPs recognition by TLRs and potentiation of TLR signaling [35]. Interestingly, these TLR-dependent events are strongly reduced when the production of uric acid/uric crystal is inhibited in mice [36]. *In vitro*, the role of immune cells as donor [12, 16], receiver [15], or transmitter [8] of bystander signals have been addressed in different studies using freshly purified blood peripheral mononuclear cells (PBMCs) [8, 12] or immortalized human cell lines [8, 15, 16]. These studies mostly assessed the induction of genotoxic effects in receiver cells exposed to bystander signaling elicited by high-dose exposure but did not investigate whether and how these bystander signals modify the ability of immune cells to be activated and respond to an inflammatory stimulus.

In this article, we compared the response of primary fibroblasts and keratinocytes from the same donors to low- and high-dose radiation exposure, and analyzed the impact of radiation-induced bystander signals elicited from these cells on the inflammatory status and response of peripheral blood mononuclear cells. Fibroblasts and keratinocytes were found to produce distinct profiles of secreted cytokines, which are not modulated after exposure to low- or high-dose radiation. However, conditioned culture media from these cells modulate PBMCs inflammatory status and activation, with slight differences according to the cell type and exposure. We concluded that radiation-induced effects have the ability to modify the functionality of immune cells, and these effects appear to be distinct for each cell lineage.

MATERIALS AND METHODS

Cell culture and reagents

Primary skin cells were a generous gift from M Martin's laboratory (CEA, Evry, France). Primary skin keratinocytes (human primary keratinocytes [HKPM]) and fibroblasts (human primary fibroblasts [HFPM]) have been isolated from biopsies from three healthy female donors, numbered 168, 170 and 172, after their informed consent, as described in [37, 38]. Cells were amplified in order to constitute banks of each cell types and donors in passage 1–2. For routine culture, and after division, fibroblasts and keratinocytes were seeded in 75 cm^2 culture flasks at a density of 5000 and 800 cells/cm^2 respectively. Three to four replicates were used in all experiments for each cell type.

Fibroblasts were cultured in DMEM (Gibco® DMEM + GlutaMAXTM, Fischer Scientific, Illkirch, France) supplemented with 10% fetal bovine serum (FBS, Eurobio Scientific, Les Ullis, France) and 1% of penicillin–streptomycin antibiotics (Gibco®, Pen Strep, Fischer Scientific, Illkirch, France). For keratinocytes, we used a semi-defined KBM-2 culture medium (Lonza Clonetics®, KBM-2 keratinocyte basal medium-2, supplemented with KGM-2 Singlequots, Ozyme, St Cyr l'Ecole, France). The cells were grown at 37°C in a humidified incubator with 5% CO_2. Medium was changed every 2 days until the cells reached 70–80% confluence. Then fibroblasts were passed in a medium containing only 0.1% FBS for 5 days before their irradiation in order to maintain most cells in a quiescent state. Nutlin-3 and lipopolysaccharide (LPS) from *E. coli* 0111:B4 were purchased from Sigma Aldrich. Nutlin-3 stock solution was prepared and diluted in dimethylsulfoxyde (DMSO). It was added for the indicated time to cells prepared as described above. Untreated cells received the same volume of DMSO.

Irradiation

Keratinocytes were at passage 2 or 3 and fibroblasts between passage 4 and 7 at the time of radiation exposure in T75 culture flasks. Their respective culture medium was renewed 24 hours before irradiation. Duplicate flasks were exposed at room temperature to low (50, 100 mGy), and high (2 Gy and 10 Gy) doses of ^{60}Co γ-rays, using the Anémone Bio irradiator of ARC-Nucléart facility (CEA-Grenoble). Dose rates were 0.034 Gy / min for low doses, and 0.524 Gy / min for high doses. Control cells (non-irradiated cells) were subjected to the same conditions as irradiated cells (sham irradiation). After irradiation, cells were incubated at 37°C in a humidified incubator with 5% CO_2, for 24 h and 48 h post exposure. In these conditions, cell mortality, measured by Trypan blue exclusion or by using an automated Scepter™ Cell Counter (Merck), never exceeded 20% after 10 Gy exposure.

Supernatants and cells collection

Supernatants from cells irradiated at the different doses were collected 24 and 48 hours after exposure to γ-rays. They were centrifuged (10 min at 1400 rpm) and filtered (0.22 μM) in order to discard cells fragments, and aliquots were then frozen at −20°C until use.

After the collection of supernatants, the cells were harvested by trypsinization after two washes in PBS. Trypsin-EDTA (Fischer Scientific, Illkirch, France) was used at 0.25% for fibroblasts or diluted to 0.05% for keratinocytes for 5 minutes at 37°C, then inactivated with FBS (20% final) and finally rinsed twice in D-PBS.

RNA purification—quantitative real-time PCR analysis

Total RNA was extracted from cells at the indicated times with the Nucleospin RNA/Protein purification kit (Macherey-Nagel, Hoerdt, France), following the manufacturer's protocol. RNA was eluted in RNAse-free water and quantified using a NanoDrop-2000 spectrophotometer.

Total RNA (0.6 to 1 μg) was converted into cDNA by priming with oligo-dT using the HS RT-PCR kit (Sigma-Aldrich France, L'lsle D'Abeau Chesnes, France) according to manufacturer's protocol and diluted to a final volume of 50 μL. A control reaction without reverse transcriptase (noRT control) was included in each series of samples.

Real-time PCR was performed in 384 well plates, in a C1000 TM thermal cycler (BioRad Life Sciences, Marnes-la-Coquette, France), in a final volume of 10 μL containing 5 μL of Lumino CT reaction mix (Sigma-Aldrich France, L'lsle D'Abeau Chesnes, France), 2 μL of cDNA diluted 1/8 and 2 μL of primers at 2 mM each. After 20 s of denaturation at 95°C, amplification was performed for 40 cycles consisting of 5 s of denaturation at 95°C and 20 s of elongation at 60°C. All reactions were carried out in triplicate. The specificity of the reactions was verified by a melting curve analysis of the products generated. Amplification of the noRT control sample was used to check the absence of contaminating genomic DNA in our RNA preparations. Data were collected and analyzed with CFX Manager 3.1 software (BioRad Life Sciences, Marnes-la-Coquette, France). We used primers designed to amplify p53-dependent stress response genes (CCNG1, DDB2, PHPT1 and CDKN1A, coding for the p21 protein) and genes coding for inflammatory mediators (CSF1, CXCL8/IL-8, IL1B, MCP-1, TGFB1 and TNFA). PCR reactions were normalized using two housekeeping genes (HPRT and 36B4). The sequence of the primers is provided in Supplementary Table S1. PCR runs were validated when the amplification of the housekeeping genes resulted in a coefficient of variation and M-values < 0.25 and < 0.5, respectively, as calculated by the CFX manager software, indicating that they are not differentially regulated in our experimental conditions.

Quantification of cytokines secretion

We simultaneously measured the secretion of IL-6, IL-8, MIP1α, GM-CSF, MCP-1 and TNFα in supernatants of control and irradiated HFPM and HKPM cells collected 24 and 48 hours post-exposure using multiplex ELISA on bead assays (Cytometric Bead Arrays, Becton-Dickinson Life Sciences-Biosciences, Le Pont-de-Claix, France) according to manufacturer's instruction. Results were analyzed using the FCAP array software (Becton-Dickinson Life Sciences-Biosciences, Le Pont-de-Claix, France), and normalized on the number of live cells in each condition.

Functional analysis of BEs

PBMCs were purified from buffy coats purchased from the French Blood Bank (Etablissement Français du Sang – Rhone-Alpes, La Tronche, France, under contract #15–2041) by density gradient using UNI-SEP tubes (NOVAmed, Eurobio Scientific, Les Ulis, France) according to manufacturer's instructions. These PBMCs were then resuspended at a concentration of 2 to 4 $\times$ 10^6 cells/mL in supernatants from control and irradiated HFPM and HKPM cells, and incubated at 37°C in a humidified incubator with 5% CO2 without or with LPS (1 ng/mL) for 20 h in 6-wells plates.

RNA prepared from PBMCs cultured in HFPM168 and HKPM168 supernatants was used to quantify the expression of cytokine (IL1B, CCL3, CXCL8/IL8) and stress response (DDB2, PHPT1, CCNG1) genes as described above. PCR results from 3 replicate experiments were analyzed with the CFX Manager 3.1 software 'volcano plot' option to compare the modulation of gene expression in PBMCs incubated in irradiated vs control HFPM and HKPM conditioned culture media, with a fold change threshold of 1.6 (arbitrarily selected) to identify genes up- or down-regulated with a P-value < 0.05 by a t-test.

Culture supernatants were harvested as above to quantify the secretion of IL-1β, IL-6, IL-8 and MCP-1 by PBMCs by FACS, using multiplex ELISA on beads assays as described above. Results were analyzed using the FCAP array software (Becton-Dickinson) and expressed normalized on cytokine secretion by PBMCs cultured in the supernatant of non-irradiated fibroblasts and keratinocytes.

Statistical analysis

Statistical analysis was performed using the Past 4.03 freeware [39]. Multiple comparisons were performed by an ANOVA test followed by Tuckey's pairwise comparison test if the data were normally distributed, or a Kruskal–Wallis test followed by a Dunn's post hoc test is the distribution was not normal. Normality was determined by the Shapiro–Wilk test. For pairwise comparisons, a t-test was performed.

RESULTS

Experimental strategy

The experimental strategy devised to address the potential modifier role of radiation-induced bystander signals produced from irradiated primary fibroblasts and keratinocytes onto the inflammatory response is depicted in Fig. 1. Primary fibroblasts and keratinocytes were exposed to γ-ray doses ranging from 0.05 to 10 Gy. These cells and their culture media were harvested 24 h and 48 h after exposition to analyze the direct effects of radiation exposure, more specifically on the expression of a panel of genes coding for inflammatory factors. This panel includes genes coding for chemokines (CSF1, CXCL8/IL-8, MCP-1) and pro- (IL1B, TNFA) and anti (TGFB1)-inflammatory cytokines to analyze the eventual regulation of a broad spectrum of potential activities on immune cells. In addition, the conditioned culture media harvested from un-irradiated control and irradiated fibroblasts and keratinocytes was used to cultivate purified PBMCs for 24 h, either in a resting state (no LPS) or activated by LPS (+LPS). We then measured the transcription of stress-response and inflammatory genes in these PBMCs, and their secretion of inflammatory cytokines to analyze the indirect effects of the exposure of fibroblasts and keratinocytes.

Modulation of stress and inflammatory responses in primary fibroblasts exposed to low- and high-dose radiation

HFPM from three different donors (donors 168, 170 and 172) were exposed to low (50 mGy, 100 mGy) and high (2 Gy, 10 Gy) doses of γ-radiation from a ^{60}Co source. Cells and their supernatants were harvested 24 and 48 hours later as described in Materials and Methods. We compared in these cells the relative expression of a panel of stress-responsive, p53-dependent genes (CCNG1, DDB2, CDKN1A, PHPT1) and of a panel of genes coding for inflammatory cytokines (CSF1, CXCL8/IL-8, MCP-1, TGFB1, IL1B, TNFA) by RT-qPCR 24 h and 48 h after exposure (Fig. 2). Low-dose exposure did not affect the relative expression any of these genes when compared to un-irradiated control cells. High-dose exposure induced a significant up regulation of the stress response genes 24 h post irradiation when compared to control or low-dose exposed cells, which was maintained or slightly increased at 48 h. The increase in gene expression is

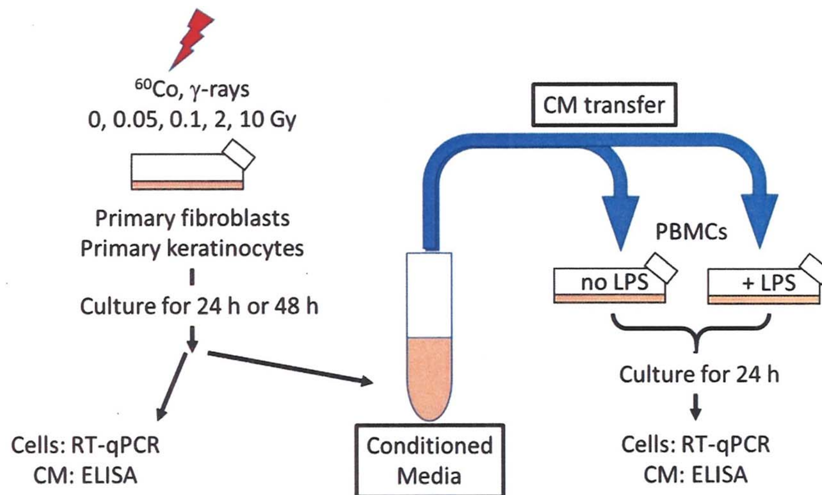

Fig. 1. Schematic representation of the experimental strategy designed to analyze the direct and bystander consequences of primary fibroblasts and keratinocytes exposure to low- and high-doses of γ-rays. Directs effects were analyzed by RT-qPCR and ELISA 24 h and 48 h after primary cells exposure (left). BEs were analyzed by RT-qPCR and ELISA after culture of resting or LPS-activated PBMCs in conditioned culture media (CM) harvested from control and irradiated primary cells.

radiation-dose dependent for DDB2 and CDKN1A at 24 h, but only for CDKN1A at 48 h. The modulation of cytokine gene expression after high-dose exposure is clearly different. The relative expression of IL-8 is downregulated in cells exposed to 10 Gy when compared to un-irradiated and 2 Gy exposed cells at 24 h and 48 h, respectively. The relative expression of MCP-1 is significantly increased only after exposure to 2 Gy and always higher than in 10 Gy exposed cells, at both 24 h and 48 h. Transcription of TGFB1 is increased after 10 Gy at both time, but after 2 Gy only at 48 h when compared to un-irradiated control cells and cells exposed to low-dose radiation. The relative expression of IL1B and TNFA was not reliably detected in these cells. Thus, in primary fibroblasts, we do not observe any transcriptional regulation of this panel of inflammatory genes after low-dose exposure, and exposure to high doses induces can result in sporadic increase or decrease of specific genes.

To find out whether the transcription of cytokine genes can be induced by a more sustained activation of the DNA damage response (DDR) mechanisms activated after radiation exposure, primary fibroblasts were treated with nutlin-3, which activates the p53 pathway by inhibiting p53-MDM2 interactions [40]. In HFPM168 cells treated with nutlin-3 (1 and 10 μM) for 6 or 24 h, we observed, as expected, a clear up-regulation of the transcription of all the stress response genes, but also of most of the inflammatory cytokine genes analyzed (Supplementary Fig. S1). A dose-dependent induction of CCNG1 and CDKN1A expression is already evident after 6 h of stimulation, and is maintained until 24 h, whereas DDB2 and PHPT1 induction become nutlin-3 dose-dependent only at that time. In contrast, transcriptional up-regulation of cytokine genes occurs only in cells treated with 10 μM nutlin-3. This increase already occurs after 6 h of culture for CSF1 and IL1B, but only after 24 h for CXCL8/IL-8 and MCP-1. The transcription of TGFB1 is not significantly affected by p53 activation. Thus, in primary fibroblasts, a strong and sustained pharmacological activation of p53 can markedly induce the transcription of inflammatory mediators genes, but exposure to low- and high-dose radiation have at most a very limited effect.

RT-PCR analysis only offers a snapshot of gene expression at the time the cells were harvested. Indeed, cytokine production can also be regulated at the post-transcriptional level and/or at the level of secretion [41]. Thus, to find out whether cytokine secretion was regulated after radiation exposure, we measured the accumulation of a series of cytokines (IL-6, IL-8, MIP1α, GM-CSF, MCP-1 and TNFα) in the conditioned culture medium of HFPM168 and HFPM170 cells exposed to low (50 and 100 mGy) and high (2 Gy) doses of radiation, 24 h and 48 post exposure. We did not consider supernatants from HFPM cells exposed to 10 Gy in these analyses to avoid an eventual interference of cytokines released rather than secreted after cell death, even though viability was always greater than 80% after 10 Gy exposure. Only IL-6, IL-8 and MCP-1 could be reliably detected. HFPM168 and HFPM170 cells secreted similar levels of IL-8 and MCP-1, but the former was found to produce more IL-6 than the latter (Supplementary Fig. S2). The secretory profiles of these three cytokines is not affected by radiation and time post exposure (Fig. 3, left) We concluded that exposure to low- or high-dose radiation does not significantly modulate the secretion of these cytokines in the 2 days following exposure.

Modulation of stress and inflammatory responses in primary keratinocytes exposed to low- and high-dose radiation

The same experiments were performed on HKPM obtained from the same donors (donors 168, 170 and 172), with the difference that only HKPM170 cells were exposed to 10 Gy. The effects of radiation on primary keratinocytes were essentially the same than in primary fibroblasts 24 h after high-dose exposure, even though the level of induction was lower. In addition, in contrast to the results obtained with fibroblasts, the up-regulation of stress genes expression is not

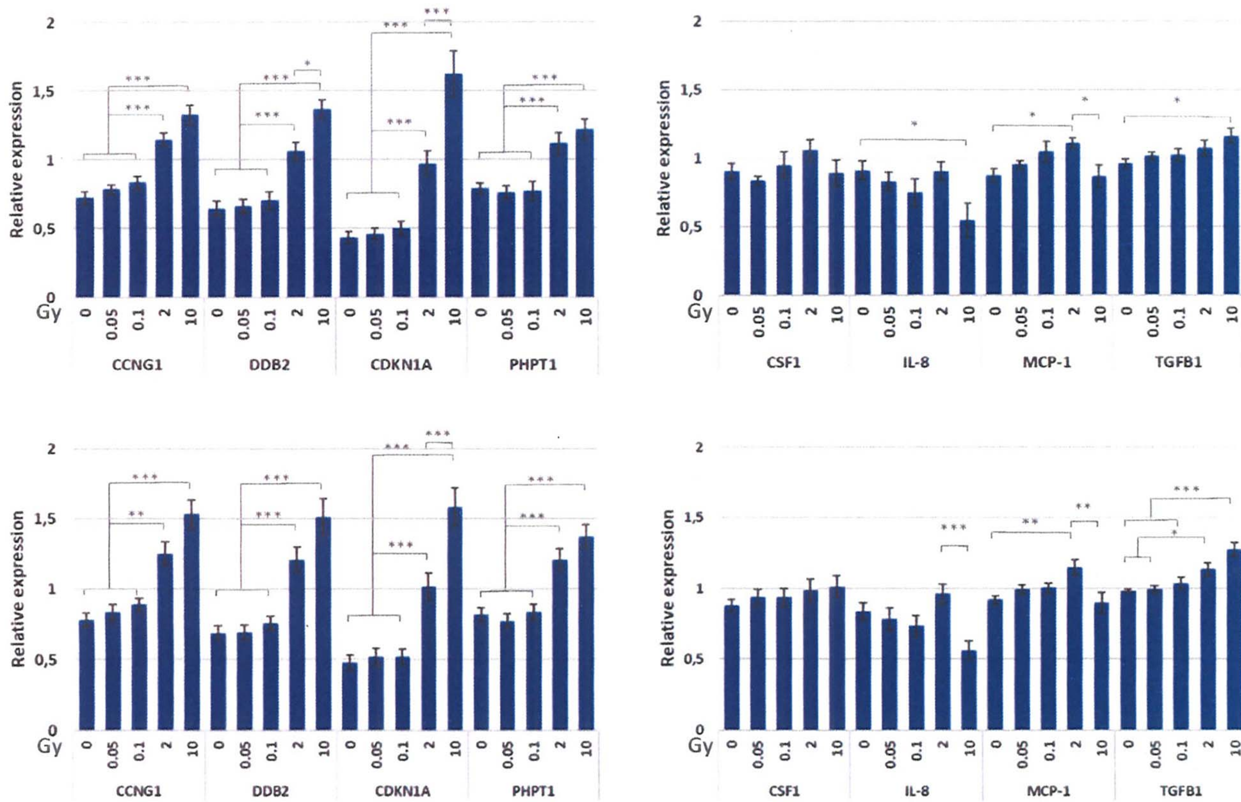

Fig. 2. Modulation of stress and inflammatory gene expression in irradiated primary fibroblasts. The expression of stress (left) and inflammatory (right) genes was analyzed by RT-qPCR 24 h (top) and 48 h (bottom) after exposure of HFPM168 ($n = 4$), HFPM170 ($n = 3$) and HFPM172 ($n = 4$) cells to 0.05, 0.1, 2 and 10 Gy of γ-radiation. The x axis (Gy) indicates the dose of radiation. Only HFPM170 and HFPM172 were exposed to 10 Gy. For each gene, the level of expression in control and exposed cells was compared by an ANOVA test if the data were normally distributed or by a Kruskal–Wallis test, if they were not. When a value of $P < 0.05$ indicated significant differences in irradiated cells, pairwise comparisons were performed with a Tukey's test, after an ANOVA test, or a Dunn's post hoc test, after a Kruskal–Wallis test. The graphs represent mean ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

maintained at day 2 post exposure. The relative expression of DDB2, CDKN1A and PHPT1 after 2 Gy exposure is for example no longer significantly higher than that of cells exposed to low-dose radiation at 48 h (Fig. 4). Unlike in fibroblasts, we could not reliably detect CSF1 transcription in keratinocytes while IL1B and TNFA genes were readily amplified. Again, radiation exposure had limited effects on cytokine gene expression. IL1B and TGFB1 expression was slightly up-regulated 24 h after 2 Gy, and that of TGFB1 after 2 and 10 Gy exposure. The relative expression of CXCL8/IL-8 and TNFA was decreased in cells exposed to high-dose radiation. These modifications were only transient as these changes were no longer visible 48 h post exposure. Thus, here again, we do not observe any modulation of the relative expression of inflammatory genes after low-dose exposure. Exposure to high doses results in up- or down-regulation of the transcription of specific genes, but, in contrast to primary fibroblasts, these changes are transient.

The expression of these genes was then analyzed in HKPM168 cells treated by low (1 μM) and high (10 μM) doses of nutlin-3 for 6 or 24 h (Supplementary Fig. S1). In contrast to the results obtained with primary fibroblasts, the expression of the stress-response genes is significantly increased only in cells treated with 10 μM nutlin-3. Furthermore, the expression of DDB2 and PHPT1 is induced only after 24 h of stimulation. A similar profile is observed for the relative expression of IL1B and TGFB1, while that of the IL-8 gene is not significantly modified in any condition, when compared to untreated cells. Thus, nutlin-3 appears to be less potent on primary keratinocytes than on primary fibroblasts. These results nonetheless show that prolonged activation of p53, but not radiation exposure, can induce the transcription of at least some of the inflammatory cytokine genes.

Finally, as for fibroblasts, we measured the accumulation of IL-6, IL-8, MIP1α, GM-CSF, MCP-1 and TNFα in the medium of HKPM168 and HKPM170 cells 24 h and 48 h after exposure to low (50 and 100 mGy) and high (2 Gy) dose radiation. HKPM168 and HKPM 170 cells were found to produce similar levels of IL-8 (Supplementary Fig. S2), whereas HKPM170 produced in

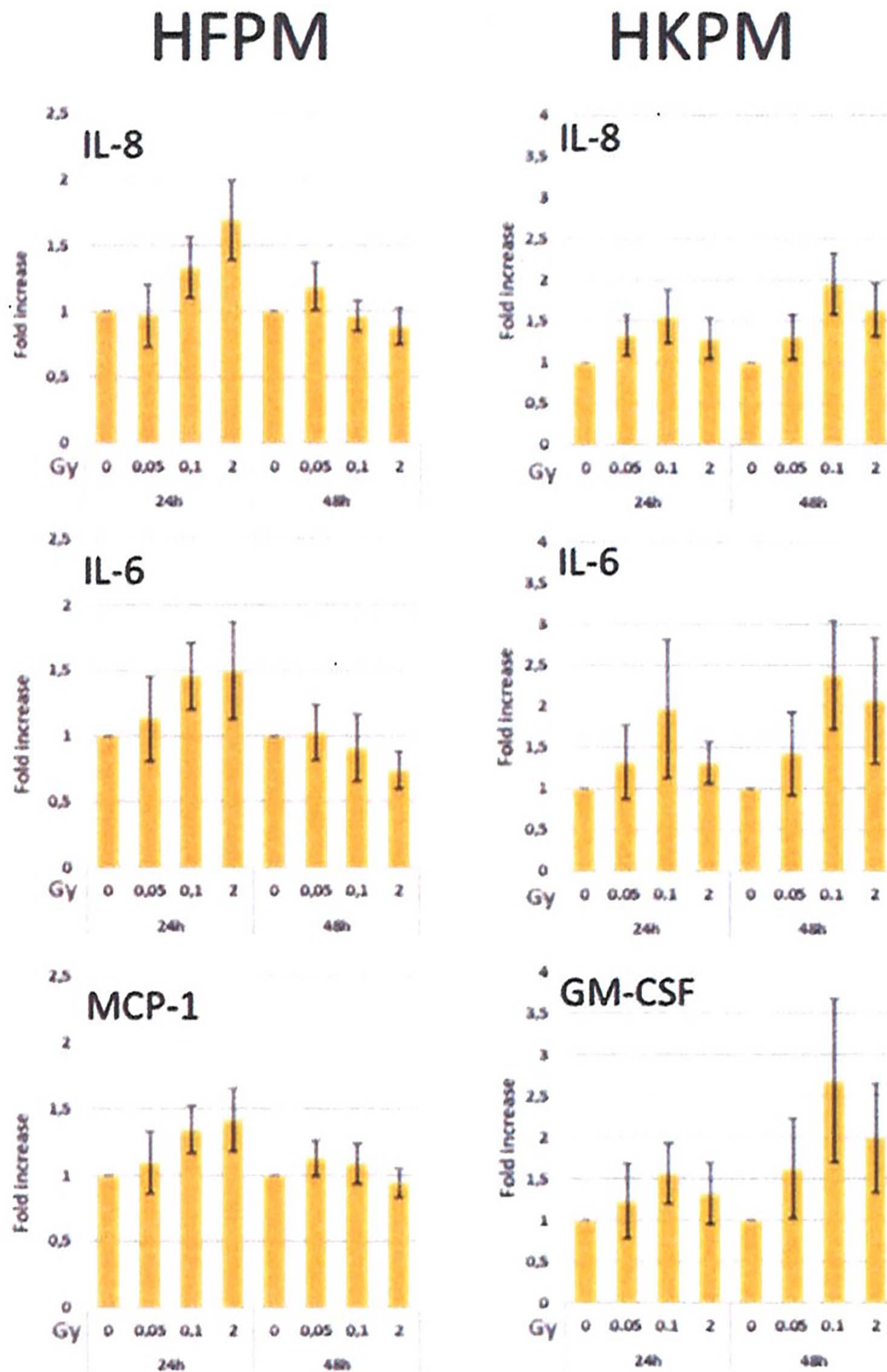

Fig. 3. Effects of radiation exposure on inflammatory cytokine secretion by primary fibroblasts and keratinocytes. The level of IL-6, IL-8, MIP1α, GM-CSF, TNFα and MCP-1 was measured in supernatants of HFPM (left) and HKPM (right) cells one and two days after exposure to the doses of radiation indicated on the x axis (Gy). Results for fibroblasts were obtained from HFPM168 ($n = 3$) and HFPM170 ($n = 3$). Results from keratinocytes were obtained from HKPM168 ($n = 3$) and HKPM170 ($n = 4$). HKPM168 was found to secrete only IL-8; MIP1α and TNFα were not detected in any condition. For each cytokine, the level of secretion in irradiated cells was compared to control cells by an ANOVA test. No significant differences were found ($P > 0.05$). The graphs represent mean $\pm$ SEM.

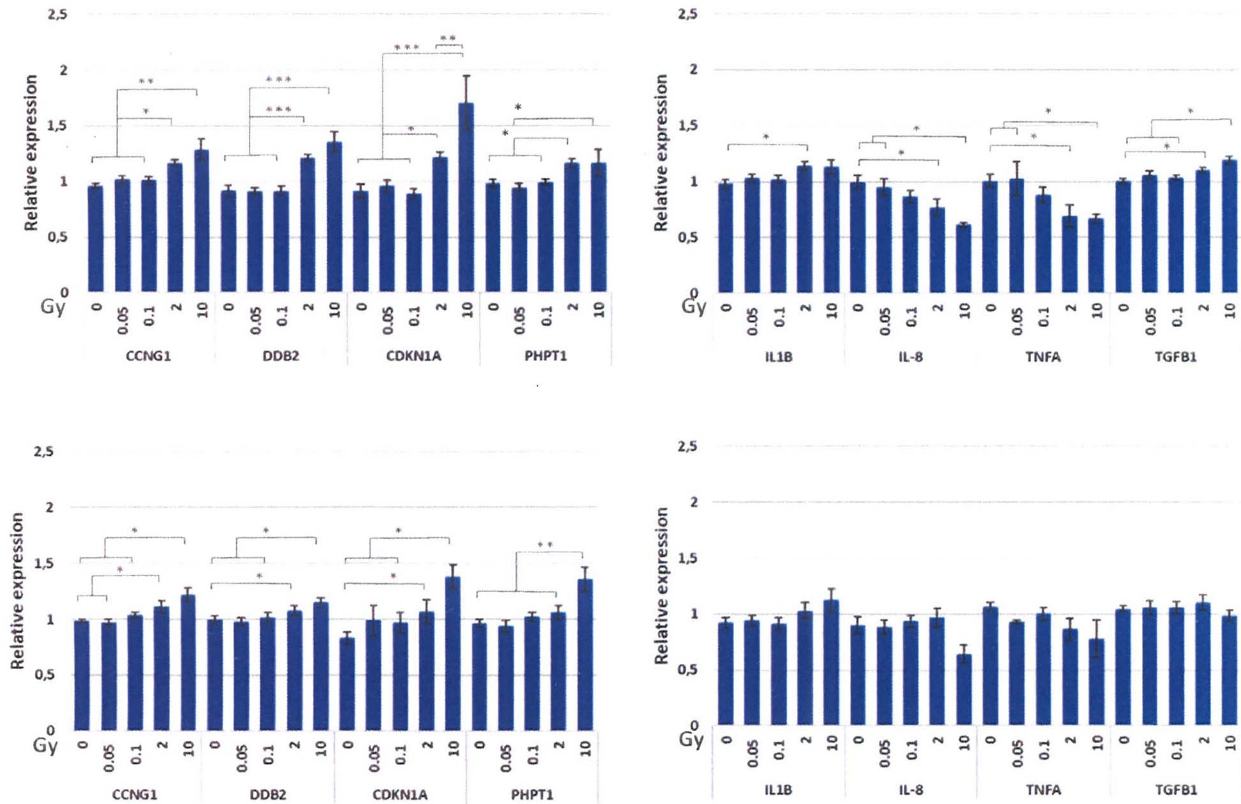

Fig. 4. Modulation of stress and inflammatory gene expression in irradiated primary keratinocytes. The expression of stress (left) and inflammatory (right) genes was analyzed by RT-qPCR 24 h (top) and 48 h (bottom) after exposure of HKPM168 ($n = 4$), HKPM170 ($n = 4$) and HKPM172 ($n = 4$) cells to 0.05, 0.1, 2 and 10 Gy of γ-radiation. Only HKPM170 and 172 cells were found to transcribe the TNFA gene at a detectable level. The x axis (Gy) indicates the dose of radiation. Only HKPM170 were exposed to 10 Gy. For each gene, the level of expression in control and exposed cells was compared by an ANOVA test if the data were normally distributed or by a Kruskal–Wallis test, if they were not. When a value of $P < 0.05$ indicated significant differences in irradiated cells, pairwise comparisons were performed with a Tukey's test, after an ANOVA test, or a Dunn's post hoc test, after a Kruskal–Wallis test. The graphs represent mean ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

addition IL-6 and GM-CSF (Fig. 3, right). None of the apparent changes observed with radiation and time post exposure are significant.

Influence of BEs on the inflammatory response of PBMCs

Even though we did not observe any significant changes in cytokine secretion by low- and high-dose irradiated primary fibroblasts and keratinocytes, we wanted to determine whether radiation-induced changes could have functional consequences and modulate the inflammatory status and/or response of bystander immune cells.

To this end, we cultured freshly purified PBMCs for 24 h in conditioned culture media harvested from control and irradiated keratinocytes and fibroblasts 24 h and 48 h after exposure to 0.05, 0.1 or 2 Gy, in absence of additional stimulation or with LPS. LPS exposure induces an inflammatory reaction and results in a strong up-regulation of cytokine production by activated PBMCs (Supplementary Fig. S3). The BEs induced by the conditioned media were assessed at the transcriptional level, by the quantification of cytokine and stress response genes expression, and by measuring the modulation of LPS-induced cytokine secretion by PBMCs. This experimental setting allows us to investigate whether and how bystander signaling affects PBMCs inflammatory status and response, i.e. their functionality.

HFPM168 supernatants harvested 24 h post 0.05 and 0.1 Gy exposure specifically reduced by 40% or more (FC < -1.6, $P < 0.05$) the transcription of IL1B ($P = 0.054$ in 0.05 Gy supernatants), CCL3 and CXCL8/IL8 genes in PBMCs when compared to non-irradiated HFPM168 (Fig. 5). Supernatants harvested from 2 Gy exposed fibroblasts only induced a reduction of CCL3 gene expression ($P = 0.051$). In 48 h HFPM168 supernatants the level of IL1B, CCL3 and CXCL8/IL8 (FC = -1.59) were coordinately down regulated in PBMCs only when fibroblasts were exposed to 0.05 Gy; IL1B expression was also found reduced (FC = -1.59) after 2 Gy exposure when compared to non-irradiated control supernatants. The transcription of DDB2, CCNG1 and PHPT1 was not modulated in PBMCs in any of these conditions. The reduction in cytokine

gene expression in irradiated HFPM168 supernatants is lost when PBMCs are stimulated with LPS (data not shown). Conditioned culture media collected from control and irradiated HKPM168 cells had no effects of inflammatory and stress response gene expression in PBMCs, in absence or in presence of LPS (Fig. 5 and data not shown). Thus, signals resulting from irradiated fibroblasts, but not keratinocytes of a same donor are able to specifically modulate the transcription of inflammatory genes in bystander immune cells.

Next, to find out whether these effects on the inflammatory status of PMBCs translate at the level of their inflammatory response, we measured their secretion of cytokines as resting PMBCs and after LPS activation. In conditioned media obtained from control and irradiated fibroblasts (HFPM168 and HFPM170), resting PBMCs produced only IL-6 and MCP-1, but at levels that did not differ from the levels produced by un-irradiated primary fibroblasts. We therefore could not conclude on the origin of these cytokines. However, their levels were not modulated in supernatants from irradiated fibroblasts (Supplementary Fig. S4). LPS-stimulated PBMCs produced MCP-1, IL-6 and IL-1β. IL-1β secretion was decreased in conditioned media collected from low- and high-dose exposed HFPM cells 24 h post irradiation when compared to non-irradiated control supernatants (Fig. 6). This modulation appears to evolve with time and/or exposure as these effects persisted only in 48 h high-dose supernatants. LPS-induced production of MCP-1 and IL-6 is not significantly affected in the same conditions, suggesting that the reduced IL-1β secretion does not result from PBMCs death.

Resting PBMCs cultured in conditioned culture media harvested from un-irradiated primary keratinocytes produced IL-6, IL-8 and MCP-1. Here again we could not ascertain the origin of IL-6 and IL-8, as the levels measured were similar to that found in keratinocyte culture media. However, similar to the situation observed with primary fibroblasts, these cytokines were not modulated in irradiated keratinocytes conditioned culture media (Supplementary Fig. S4). LPS-activated PBMCs cultured in conditioned media harvested from HKPM168, HKPM170 and HKPM172 cells produced IL-8 in addition to high levels of MCP-1, IL-6 and IL-1β in control (Supplementary Fig. S3) and irradiated keratinocyte supernatants. Surprisingly, given the absence of effects of HKPM168 supernatants on PBMCs inflammatory status, the LPS-induced production of IL-6, IL-8 and IL-1β was decreased in conditioned media from keratinocytes exposed to 2 Gy when compared to supernatants from control and/or low-dose irradiated cells, in supernatants collected at 48 h (IL-6, IL-1β) or at 24 and 48 h (IL-8) (Fig. 6). In contrast, conditioned culture media harvested from low-dose exposed keratinocytes could either enhance or reduce the production of cytokines by PBMCs following LPS stimulation. Supernatants collected 24 h after 50 mGy exposure enhance the LPS-induced IL-8 secretion by PBMCs, while those collected 48 h after 100 mGy exposure reduce LPS-induced IL-1β production. Thus, whereas exposure of keratinocytes to high-dose radiation impairs the response of PBMCs to LPS, the effects of low-dose radiation are mixed, with both potentiation and inhibition of cytokine secretion, when compared to non-irradiated keratinocytes. The overall result is a qualitatively different profile of cytokine secretion by PBMCs upon LPS stimulation.

DISCUSSION

We compared the cellular and bystander responses induced in/from primary fibroblasts and keratinocytes by low and high radiation doses with the aim to determine whether and how radiation exposure can modulate the functionality of immune cells. Our results show that radiation exposure elicits different patterns of responses in fibroblasts and keratinocytes, and that radiation-induced bystander signals have functional consequences as they affect the inflammatory status and response of purified PBMCs.

Radiation-induced modulation of gene expression in skin cells after low- and/or high-dose exposure have already been largely documented in primary fibroblasts [42–45], keratinocytes [45, 46] and skin/skin models [47–50]. Cell–cell signaling pathways are for example modulated in primary fibroblasts or skin plugs after low doses, while the response to higher doses was dominated by changes in cell proliferation and apoptosis pathways [43, 49, 50]. In a different study, pathways related to cancer and metabolism were found induced only in high, but not low-dose exposed primary fibroblasts [42]. Specific genes were found up- or down-regulated differently over time in low-dose exposed keratinocytes [46]. Collectively, these results illustrate the diversity and complexity of the transcriptional responses elicited by ionizing radiation in different experimental settings. In addition, these responses may also depend on inter-individual variability, as shown by the comparison of the response elicited in skin biopsies from different patients after *in vivo* exposure to 100 mGy, with as many as four out of eight patients showing no significant changes in the expression of genes belonging to the cytokine and inflammation pathways [48]. Here, we compared the expression of a selected set of stress response and inflammatory genes in primary fibroblasts and keratinocytes established from the same donors after low- and high-dose exposure. While the relative expression of stress response genes was always up-regulated after high-dose exposure, expression of some of the inflammatory genes was unchanged, induced or repressed in the same samples (Figs 2 and 4). It is unlikely that the reduced levels of IL-8 and TNFA transcripts in high-dose exposed fibroblasts and/or keratinocytes result from radiation-induced cell death, as, first, mortality never exceeds 20% after 10 Gy exposure, and second, in that case, a similar reduction would be observed for all genes analyzed. This different behavior in response to high-dose radiation exposure therefore suggests that the mechanisms responsible for the modulation of the two sets of stress and inflammatory genes are different in irradiated cells and are not governed solely by the activation of the ATM-dependent DDR.

The situation is however different in response to nutlin-3 stimulation, which results in the induction of these genes (Supplementary Fig. S1). These results strongly suggest that a strong and sustained activation of p53 activity is able to stimulate the transcription of inflammatory genes in primary fibroblasts and keratinocytes including CXCL8/IL-8, suggesting that its down-regulation in irradiated cells is not directly under the control of p53 activation. The level of p53 activation after exposure to 2 and 10 Gy might not be 'high' enough to promote a strong induction of inflammatory genes. They also suggest that beside the radiation-induced DDR, one or more additional mechanism(s) is/are induced that can repress inflammatory gene transcription or counteract p53 transcriptional activity in irradiated primary fibroblasts and keratinocytes. These mechanisms may for

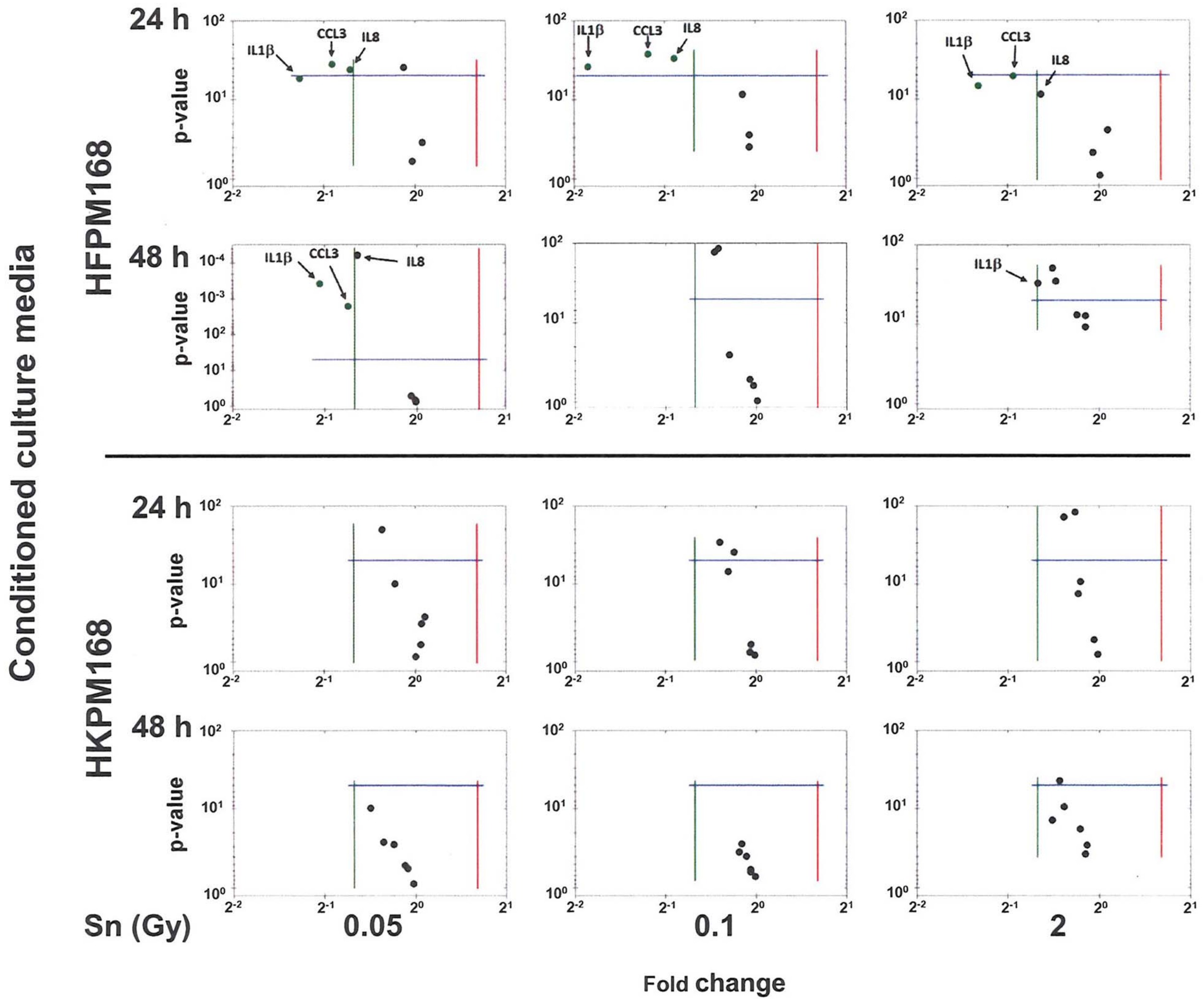

Fig. 5. Influence of BEs on PBMCs inflammatory status. The level of expression of IL1B, CCL3, CXCL8/IL8, CCNG1, DDB2 and PHPT1 genes was measured by RT-qPCR in purified PBMCs cultured 24 h in conditioned culture media harvested from non-irradiated and irradiated HFPM168 (top) and HKPM168 (bottom) cells harvested 24 h and 48 h post exposure. These volcano plots show the relative normalized expression of these 6 genes in PBMCs cultured in supernatants collected from cells exposed to 0.05 (left), 0.1 (center) and 2 (right) Gy, compared to their expression in non-irradiated control supernatants. The green and red lines represent a fold change (FC) of 1.6 time, respectively, and the blue line the threshold *P*-value = 0.05, calculated by a t-test. *n* = 3 for PBMCs cultured in each of eh HFMP and HKPM supernatants.

example involve the restraining of NF-κB activity by ATM [51], which would occur concurrently of p53 activation by radiation. Irrespective of the exact nature of these mechanisms, it is interesting to note that the nutlin-3-induced transcriptional regulation of TGFB1, CXCL8/IL-8 and IL1B genes is different in HFPM and HKPM cells, illustrating here again differences in the regulation of their expression in primary fibroblasts and keratinocytes from a same donor. It will be of interest to identify other genes coding for immune factors similarly regulated, and the molecular mechanisms involved, to better understand the effects exposure and/or p53 activation on immune functions.

Different cytokines have been involved in the transmission of BEs [5, 22, 23, 52].Our analysis of a limited number of selected cytokines illustrates qualitative and quantitative differences in cytokine production by primary fibroblasts and keratinocytes: MCP-1 and GM-CSF are selectively produced only by HFPM and HKPM cells, respectively; keratinocytes secrete more IL-8 than fibroblasts, and, conversely, fibroblasts produce more IL-6 than keratinocytes (Fig. 3). These differences may explain that the conditioned culture media harvested from fibroblasts and keratinocytes have different effects on the inflammatory status of bystander PBMCs, as only conditioned culture media harvested from irradiated fibroblasts down-regulate their expression of inflammatory genes. However, irradiated fibroblasts and irradiated keratinocytes supernatants are both able to regulate the secretion of inflammatory cytokines from LPS-activated PBMCs,

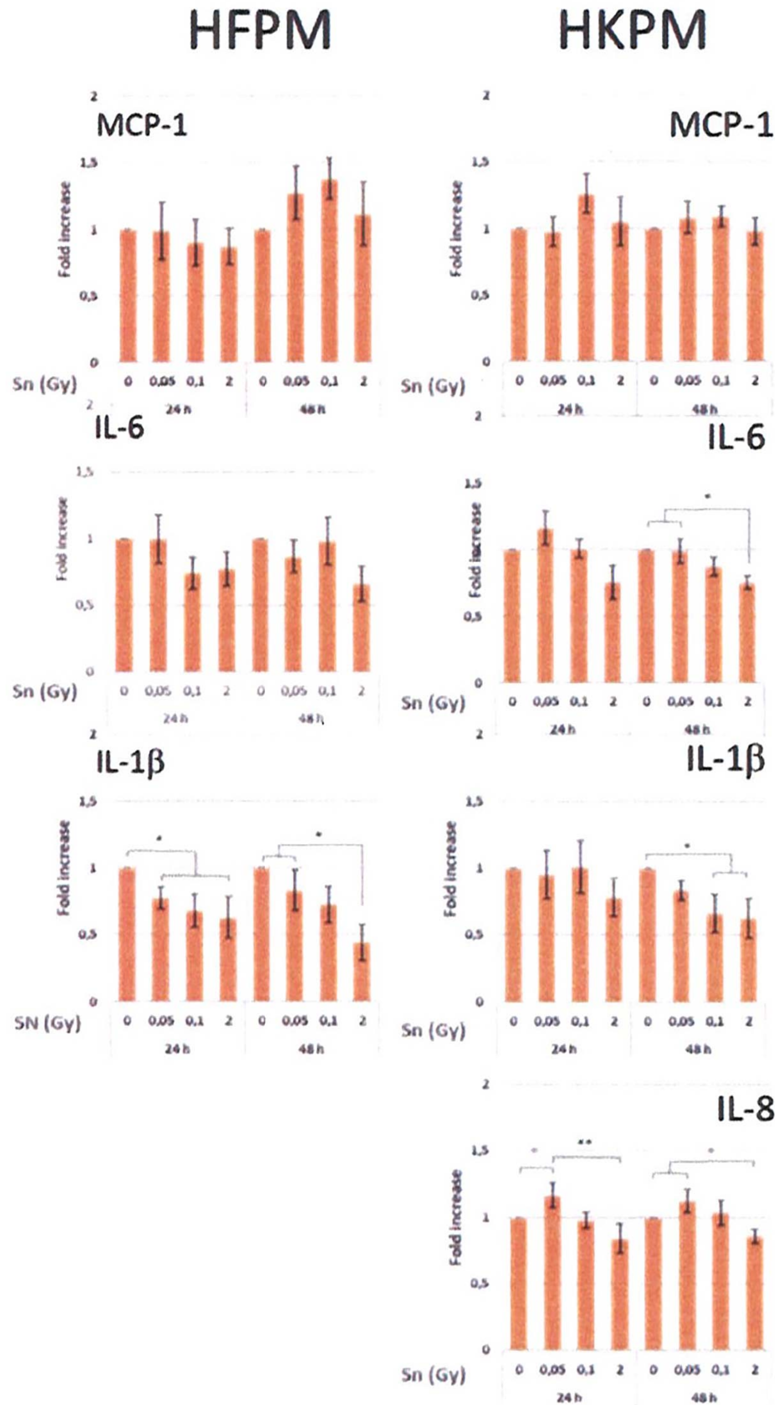

Fig. 6. Influence of BEs on PBMCs activation. Quantification of cytokine secretion by PBMCs stimulated by LPS (1 ng/mL) in control and irradiated primary fibroblasts (left) and keratinocytes (right). For HFPM cells, histograms represent the result of supernatant transfer experiments performed with conditioned culture media harvested 24 h and 48 h after exposure of HFPM168 ($n = 3$) and HFPM170 ($n = 3$ for IL-6 and $n = 4$ for IL-8 and MCP-1) to the indicated radiation doses. For HKPM cells, these experiments were performed with conditioned culture media harvested from HKPM168 ($n = 3$), HKPM170 ($n = 3$) and HKPM172 ($n = 4$) exposed to the same radiation doses. The x axis [Sn(Gy)] indicates the dose of radiation received by HFPM and HKPM cells before harvesting the conditioned culture medium. For each cytokine, the level of secretion in the different supernatants was normalized to that of PBMCs cultured in conditioned media harvested from corresponding non-irradiated HFPM or HKPM cells. Comparisons were performed by a Kruskal–Wallis test followed by pairwise comparisons by a Dunn's post hoc test when $P < 0.05$. The graphs represent mean ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

with slightly different outcomes for IL-1β and IL-6. Furthermore, only keratinocytes, but not fibroblasts, support the secretion of IL-8 by LPS-stimulated PBMCs. Thus, the secretory profiles of primary fibroblasts and keratinocytes are different, and BEs mediated by irradiated HFPM and HKPM cells on resting and activated immune cells are different and distinctly modulated by radiation exposure.

Supernatants from high-dose exposed HFPM and HKPM cells always reduce LPS-activated secretion of pro-inflammatory cytokines by PBMCs. In contrast, HKPM supernatants collected 24 h after exposure to 0.05 Gy induce a small but significant increase in LPS-induced IL-8 secretion (Fig. 6). High-dose-induced bystander signals appear to impair some aspects of the stimulation of PBMCs via activation of TLR4, the LPS receptor, while the effects of low-dose radiation can be more diverse.

Thus, bystander signaling may result in an imbalanced TLR4-dependent inflammatory response, with an increase or a decrease of only selected cytokines. The inflammatory response is a highly organized, self-limiting process ending in a resolution phase which culminates in the restoration of tissue homeostasis. The transitions between inflammation initiation, amplification and resolution are orchestrated by successive waves of pro-inflammatory and anti-inflammatory, pro-resolving factors [53–55]. In this context, bystander-induced qualitative and quantitative changes in the secretion of some, but not all, cytokines results in qualitative changes in the spectrum of secreted inflammatory mediators produced by activated immune cells. These changes may, in the long run, hamper the unfolding of the reaction resulting from TLR4 activation and modify or postpone its resolution, potentially resulting in un-resolved, inflammation or maladapted tissue homeostasis [56]. These outcomes have been linked with numerous adverse conditions [57], including the propagation of DNA damage to un-irradiated tissues following local irradiation in mice [58, 59]. Radiation-induced BEs may therefore participate in the deleterious, unwanted effects of radiation exposure, including tumor-promoting inflammation [60].

Previous work suggested that exposition of purified blood monocytes or mononuclear cells to low- and high-dose radiation could activate or potentiate TLR4-induced cytokine secretion [61, 62]. Our results now suggest that bystander signals received from both low- and high- dose exposed neighboring cells could modify the outcome of such TLR4 activation, for example after the migration of monocytes into tissues upon radiation injury. These interferences might for example impair the functional differentiation of monocytes into inflammatory macrophages and contribute to the creation of an inflammatory environment prone to support unwanted pathophysiological events [36]. However, at that time, we can only speculate on the eventual different outcomes of low- and high-dose elicited bystander signals.

It is generally admitted that low-dose radiation induce an inflammatory/tissue repair response whereas higher doses activate a p53-dependent response [see for example, 49, 62], and that bystander responses are most important after low to intermediate but not high-dose exposure (2, 5, 18, 29). This dichotomy probably raises from the fact that the cellular response to high-dose exposure is dominated by the activation of the ATM/p53 pathways during the DDR, which strongly determines cell fate and may therefore hinder the effects of bystander signals. In contrast, low-dose radiation may activate several types of stress responses, including inflammatory, oxidative, DNA damage and recovery/tissue repair responses that may be coordinated by different radiation-responsive transcription factors [49]. Cell fate is therefore the resultant of the interactions between these different pathways and, if none of them is overtly dominant [47, 49], the influence of modifiers released post radiation exposure, including cytokines, soluble mediators and microvesicles, is more easily evidenced. Here, supernatant transfer experiments allowed us to show that both low- and high-dose induced bystander signals elicited from irradiated primary fibroblasts and keratinocytes have functional consequences on PBMCs inflammatory status and activation. Furthermore, radiation-induced BEs do not result from a 'generic' radiation-induced cellular stress response, as they appear to be distinct for each cell lineage. Some of the mediators responsible for these effects are selectively produced, and, for those that are common, they are produced in different quantities and/or modulated with different kinetics and dose-response patterns in both cell types.

ACKNOWLEDGMENTS

This article is dedicated to the memory of our friend and colleague Catherine Aude-Garcia.

SUPPLEMENTARY DATA

Supplementary data is available at *RADRES Journal* online.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

FUNDING

This work was supported by financing from Electricité de France (EDF) through the Comité de Programme Protection de l'Homme et de l'Environnement (CPPHE) - GGP V3–101 'Sciences du Vivant'; GRAL, a program from the Chemistry Biology Health (CBH) Graduate School of University Grenoble Alpes (grant number ANR-17-EURE-0003); and in part by the LABEX PRIMES (grant number ANR-11-LABX-0063).

REFERENCES

1. Huang RX, Zhou PK. DNA damage response signaling pathways and targets for radiotherapy sensitization in cancer. *Signal Transduct Target Ther* 2020;5:60.
2. Lomax ME, Folkes LK, O'Neill P. Biological consequences of radiation-induced DNA damage: relevance to radiotherapy. *Clin Oncol (R Coll Radiol)* 2013;25:578–85.
3. Nagasawa H, Little JB. Induction of sister chromatid exchanges by extremely low doses of alpha-particles. *Cancer Res* 1992;52:6394–6.
4. Morgan WF, Sowa MB. Non-targeted effects induced by ionizing radiation: mechanisms and potential impact on radiation induced health effects. *Cancer Lett* 2015;356:17–21.
5. Prise KM, O'Sullivan JM. Radiation-induced bystander signalling in cancer therapy. *Nat Rev Cancer* 2009;9:351–60.

6. Wright EG. Manifestations and mechanisms of non-targeted effects of ionizing radiation. *Mutat Res* 2010;687:28–33.
7. Al-Mayah A, Bright S, Chapman K *et al.* The non-targeted effects of radiation are perpetuated by exosomes. *Mutat Res* 2015;772:38–45.
8. Fu J, Zhu L, Tu W *et al.* Macrophage-mediated bystander effects after different irradiations through a p53-dependent pathway. *Radiat Res* 2020;193:119–29.
9. Belyakov OV, Mitchell SA, Parikh D *et al.* Biological effects in unirradiated human tissue induced by radiation damage up to 1 mm away. *Proc Natl Acad Sci U S A* 2005;102:14203–8.
10. Al-Mayah AH, Irons SL, Pink RC *et al.* Possible role of exosomes containing RNA in mediating nontargeted effect of ionizing radiation. *Radiat Res* 2012;177:539–45.
11. Ariyoshi K, Miura T, Kasai K *et al.* Radiation-induced bystander effect is mediated by mitochondrial DNA in exosome-like vesicles. *Sci Rep* 2019;9:9103.
12. Moertl S, Buschmann D, Azimzadeh O *et al.* Radiation exposure of peripheral mononuclear blood cells alters the composition and function of secreted extracellular vesicles. *Int J Mol Sci* 2020;21:2336.
13. Szatmari T, Persa E, Kis E *et al.* Extracellular vesicles mediate low dose ionizing radiation-induced immune and inflammatory responses in the blood. *Int J Radiat Biol* 2019;95:12–22.
14. Blyth BJ, Sykes PJ. Radiation-induced bystander effects: what are they, and how relevant are they to human radiation exposures? *Radiat Res* 2011;176:139–57.
15. Cary LH, Noutai D, Salber RE *et al.* Interactions between endothelial cells and T cells modulate responses to mixed neutron/gamma radiation. *Radiat Res* 2014;181:592–604.
16. Xiao L, Liu W, Li J *et al.* Irradiated U937 cells trigger inflammatory bystander responses in human umbilical vein endothelial cells through the p38 pathway. *Radiat Res* 2014;182:111–21.
17. Hargitai R, Kis D, Persa E *et al.* Oxidative stress and gene expression modifications mediated by extracellular vesicles: an in vivo study of the radiation-induced bystander effect. *Antioxidants (Basel)* 2021;10:156.
18. Portess DI, Bauer G, Hill MA *et al.* Low-dose irradiation of nontransformed cells stimulates the selective removal of precancerous cells via intercellular induction of apoptosis. *Cancer Res* 2007;67:1246–53.
19. Sokolov M, Neumann R. Global gene expression alterations as a crucial constituent of human cell response to low doses of ionizing radiation exposure. *Int J Mol Sci* 2015;17:55.
20. Lepleux C, Marie-Brasset A, Temelie M *et al.* Bystander effectors of chondrosarcoma cells irradiated at different LET impair proliferation of chondrocytes. *J Cell Commun Signal* 2019;13: 343–56.
21. Zhou H, Ivanov VN, Lien YC *et al.* Mitochondrial function and nuclear factor-kappaB-mediated signaling in radiation-induced bystander effects. *Cancer Res* 2008;68:2233–40.
22. Lorimore SA, Chrystal JA, Robinson JI *et al.* Chromosomal instability in unirradiated hemaopoietic cells induced by macrophages exposed in vivo to ionizing radiation. *Cancer Res* 2008;68:8122–6.
23. Shao C, Folkard M, Prise KM. Role of TGF-β1 and nitric oxide in the bystander response of irradiated glioma cells. *Oncogene* 2008;27:434–40.
24. Lyng FM, Howe OL, McClean B. Reactive oxygen species-induced release of signalling factors in irradiated cells triggers membrane signalling and calcium influx in bystander cells. *Int J Radiat Biol* 2011;87:683–95.
25. Chevalier F, Hamdi DH, Saintigny Y *et al.* Proteomic overview and perspectives of the radiation-induced bystander effects. *Mutat Res Rev Mutat Res* 2015;763:280–93.
26. Zhang Q, Matzke M, Schepmoes AA *et al.* High and low doses of ionizing radiation induce different secretome profiles in a human skin model. *PLoS One* 2014;9:e92332.
27. Yentrapalli R, Merl-Pham J, Azimzadeh O *et al.* Quantitative changes in the protein and miRNA cargo of plasma exosome-like vesicles after exposure to ionizing radiation. *Int J Radiat Biol* 2017;93:569–80.
28. Kis D, Persa E, Szatmari T *et al.* The effect of ionising radiation on the phenotype of bone marrow-derived extracellular vesicles. *Br J Radiol* 2020;93:20200319.
29. Belyakov OV, Malcolmson AM, Folkard M *et al.* Direct evidence for a bystander effect of ionizing radiation in primary human fibroblasts. *Br J Cancer* 2001;84:674–9.
30. Muller K, Meineke V. Radiation-induced mast cell mediators differentially modulate chemokine release from dermal fibroblasts. *J Dermatol Sci* 2011;61:199–205.
31. Rzeszowska-Wolny J, Herok R, Widel M *et al.* X-irradiation and bystander effects induce similar changes of transcript profiles in most functional pathways in human melanoma cells. *DNA Repair (Amst)* 2009;8:732–8.
32. Candeias SM, Testard I. The many interactions between the innate immune system and the response to radiation. *Cancer Lett* 2015;368:173–8.
33. Chen GY, Nunez G. Sterile inflammation: sensing and reacting to damage. *Nat Rev Immunol* 2010;10:826–37.
34. Rider P, Voronov E, Dinarello CA *et al.* Alarmins: feel the stress. *J Immunol* 2017;198:1395–402.
35. Tajima G, Delisle AJ, Hoang K *et al.* Immune system phenotyping of radiation and radiation combined injury in outbred mice. *Radiat Res* 2013;179:101–12.
36. Stoecklein VM, Osuka A, Ishikawa S *et al.* Radiation exposure induces inflammasome pathway activation in immune cells. *J Immunol* 2015;194:1178–89.
37. Alexaline MM, Trouillas M, Nivet M *et al.* Bioengineering a human plasma-based epidermal substitute with efficient grafting capacity and high content in clonogenic cells. *Stem Cells Transl Med* 2015;4:643–54.
38. Fortunel NO, Cadio E, Vaigot P *et al.* Exploration of the functional hierarchy of the basal layer of human epidermis at the single-cell level using parallel clonal microcultures of keratinocytes. *Exp Dermatol* 2010;19:387–92.
39. Hammer O, Harper DAT, Ryna PD. PAST: paleontological statistics software package for education and data analysis. *Paleontologia Electronica* 2001;4:1–9.

40. Vassilev LT, Vu BT, Graves B *et al.* In vivo activation of the p53 pathway by small-molecule antagonists of MDM2. *Science* 2004;303:844–8.
41. Murray RZ, Stow JL. Cytokine secretion in macrophages: SNAREs, Rabs, and membrane trafficking. *Front Immunol* 2014;5:538.
42. Brackmann LK, Poplawski A, Grandt CL *et al.* Comparison of time and dose dependent gene expression and affected pathways in primary human fibroblasts after exposure to ionizing radiation. *Mol Med* 2020;26:85.
43. Ding LH, Shingyoji M, Chen F *et al.* Gene expression profiles of normal human fibroblasts after exposure to ionizing radiation: a comparative study of low and high doses. *Radiat Res* 2005;164:17–26.
44. Fujimori A, Okayasu R, Ishihara H *et al.* Extremely low dose ionizing radiation up-regulates CXC chemokines in normal human fibroblasts. *Cancer Res* 2005;65:10159–63.
45. Warters RL, Packard AT, Kramer GF *et al.* Differential gene expression in primary human skin keratinocytes and fibroblasts in response to ionizing radiation. *Radiat Res* 2009;172:82–95.
46. Franco N, Lamartine J, Frouin V *et al.* Low-dose exposure to gamma rays induces specific gene regulations in normal human keratinocytes. *Radiat Res* 2005;163:623–35.
47. Berglund SR, Rocke DM, Dai J *et al.* Transient genome-wide transcriptional response to low-dose ionizing radiation in vivo in humans. *Int J Radiat Oncol Biol Phys* 2008;70:229–34.
48. Goldberg Z, Rocke DM, Schwietert C *et al.* Human in vivo dose-response to controlled, low-dose low linear energy transfer ionizing radiation exposure. *Clin Cancer Res* 2006;12:3723–9.
49. Mezentsev A, Amundson SA. Global gene expression responses to low- or high-dose radiation in a human three-dimensional tissue model. *Radiat Res* 2011;175:677–88.
50. Yunis R, Albrecht H, Kalanetra KM *et al.* Genomic characterization of a three-dimensional skin model following exposure to ionizing radiation. *J Radiat Res* 2012;53:860–75.
51. Sabatel H, Di Valentin E, Gloire G *et al.* Phosphorylation of p65(RelA) on Ser(547) by ATM represses NF-kappaB-dependent transcription of specific genes after genotoxic stress. *PLoS One* 2012;7:e38246.
52. Wang H, Yu KN, Hou J *et al.* Radiation-induced bystander effect: early process and rapid assessment. *Cancer Lett* 2015;356:137–44.
53. Medzhitov R, Horng T. Transcriptional control of the inflammatory response. *Nat Rev Immunol* 2009;9:692–703.
54. Serhan CN, Brain SD, Buckley CD *et al.* Resolution of in flammation: state of the art, definitions and terms. *FASEB J* 2007;21:325–32.
55. Serhan CN, Chiang N, Van Dyke TE. Resolving inflammation: dual anti-inflammatory and pro-resolution lipid mediators. *Nat Rev Immunol* 2008;8:349–61.
56. Feehan KT, Gilroy DW. Is resolution the end of inflammation? *Trends Mol Med* 2019;25:198–214.
57. Balkwill F, Charles KA, Mantovani A. Smoldering and polarized inflammation in the initiation and promotion of malignant disease. *Cancer Cell* 2005;7:211–7.
58. Lobachevsky PN, Ventura J, Giannakandropoulou L *et al.* A functional immune system is required for the systemic genotoxic effects of localized irradiation. *Int J Radiat Oncol Biol Phys* 2019;103:1184–93.
59. Redon CE, Dickey JS, Nakamura AJ *et al.* Tumors induce complex DNA damage in distant proliferative tissues in vivo. *Proc Natl Acad Sci U S A* 2010;107:17992–7.
60. Heeran AB, Berrigan HP, O'Sullivan J. The radiation-induced bystander effect (RIBE) and its connections with the hallmarks of cancer. *Radiat Res* 2019;192:668–79.
61. El-Saghire H, Michaux A, Thierens H *et al.* Low doses of ionizing radiation induce immune-stimulatory responses in isolated human primary monocytes. *Int J Mol Med* 2013;32:1407–14.
62. Menendez D, Shatz M, Azzam K *et al.* The toll-like receptor gene family is integrated into human DNA damage and p53 networks. *PLoS Genet* 2011;7:e1001360.