Comparison of X-ray and alpha particle effects on a human cancer and endothelial cells: survival curves and gene expression profiles

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Abstract

BACKGROUND AND PURPOSE: Tumours are now considered as complex tissues including endothelial cells of the tumour vasculature, which can decrease radiotherapy efficacy. It is thus important to better characterize the response of both types of cells to irradiation. This study investigated the effects of X-ray and alpha particle irradiation on cancer and endothelial cells.

MATERIALS AND METHODS: A549 non-small-cell lung adenocarcinoma cells and human endothelial cells (EC) were exposed to X-rays or alpha particles. Responses were studied by clonogenic assays and nuclei staining. A gene expression study was performed by using Taqman low density array and the results were validated by qRT-PCR and ELISA.

RESULTS: The relative biological effectiveness of alpha particles was estimated to be 5.5 and 4.6 for 10% survival of A549 cells and EC respectively. Nuclei staining indicated that mitotic catastrophe was the main type of cell death induced by X-rays and alpha particles. Both ionizing radiations induced the overexpression of genes involved in cell growth, inflammation and angiogenesis.

CONCLUSIONS: Alpha particle irradiations are more effective than X-rays. The gene expression changes observed in both cell types after alpha particle or X-ray exposure showed possible crosstalk between both cell types that may induce the development of radioresistance.

INTRODUCTION

More than 50% of all cancer patients benefit from a superior clinical outcome with at least one session of radiation therapy during their treatment [1]. However, radiotherapy also causes side effects due to radiation exposure of healthy tissues surrounding the tumour.

One way to improve radiotherapy is to maximize the dose at the tumour location while limiting the damages to healthy tissues. This can be achieved by the use of hadrontherapy instead of conventional X-ray therapy. Hadrontherapy uses particle beams while conventional radiotherapy uses photons. The dose distribution of particles allows a precise targeting of the tumour volume while sparing surrounding normal tissues. Indeed, the photons energy deposition is maximal close to the entrance in the tissues and decreases exponentially with depth. In contrast, the maximum energy deposition of light or heavy ions occurs within the Bragg peak at a depth depending on the energy of the particle [2]. The dose deposition before this peak is low and almost non-significant after it [3]. Thus, the dose delivered to healthy tissues surrounding the tumour can be minimized.

Another advantage of charged particles over X-rays is their higher linear energy transfer (LET). Compared to sparsely ionizing radiation like X-rays, high-LET particles have a higher relative biological effectiveness (RBE) because they produce an intense ionization along their track and cause severe damages to DNA which are more difficult to repair [4]. Moreover sparsely ionizing radiation effectiveness is strongly dependent on the cell cycle position. This variation of cell sensitivity with cell cycle position and proliferative status is considerably reduced with high-LET radiation. Moreover, hypoxia occurs in a lot of solid tumours and leads to radioresistance [5]. As high-LET radiation effectiveness is less dependent upon oxygen, hadrontherapy is more suitable to treat hypoxic tumours. Nonetheless, response of the tumour vasculature to radiation therapy is also playing an important role in the tumour response to radiation. Indeed, as tumours need nutrients and oxygen supply to grow,

damaging the tumour vasculature with radiation can lead to an enhanced tumour cell killing [6]. However, tumour cells can actively protect tumour-associated endothelial cells after radiation therapy by secreting growth factors and cytokines that will reduce vasculature damages thereby limiting the treatment response [7].

For these reasons, it is important to understand mechanisms of radioresistance in both tumour and endothelial cells after irradiation in order to develop new strategies to increase radiotherapy efficacy. As hadrontherapy seems to be promising, we decided to investigate the radiation responses of human cancer and endothelial cells after irradiation with alpha particles. We compared these results to conventional X-ray irradiation.

MATERIALS AND METHODS

Cell culture

A549 cells, HeLa cells, MCF-7 cells and human endothelial EAhy926 cells were sub-cultured in 75-cm² polystyrene flasks (Costar) respectively in modified Eagle's medium, Dulbecco's Modified Eagle Medium (1 g/l d-glucose, Invitrogen) and Dulbecco's Modified Eagle Medium (4.5 g/l d-glucose, Invitrogen) for the two latters, all supplemented with 10% fetal calf serum (Invitrogen). EAhy926 cells are derived from fusion of human umbilical vein endothelial cells (HUVEC) with A549 cells. All cell types were cultured under an atmosphere containing 5% CO₂.

X-ray irradiation

Twenty-four hours before irradiation, cells were plated in 25-cm² polystyrene flasks (Costar) to obtain a confluent monolayer for the irradiation. They were irradiated at room temperature using an X-ray generator (RT250, Philips Medical Systems) at a dose rate of 0.855 Gy/min in

serum-free CO₂-independent medium (Invitrogen) supplemented with 1 mM L-glutamine (Sigma).

α -particle irradiation

A 2 MV Tandem accelerator (High Voltage Engineering Europa) was used to obtain a ²⁺He homogenous broad beam. The experimental set-up and the irradiation procedure are described elsewhere [8]. The linear energy transfer (LET) of the alpha particles was set up to ~100 keV/ μ m because it corresponds to their maximum relative biological effectiveness (RBE) and the dose rate was 1 Gy/min.

Clonogenic assay

Directly after irradiation, cells were trypsinized, counted and replated for clonogenic survival assay at appropriate cell numbers in 6-well plates. The entire procedure after X-ray and alpha particle irradiations has been previously described [8]. As EC could not grow properly when plated at low densities, we used fresh medium plus 10% serum supplemented with conditioned medium 50% as recommended by Franken et al. [9]. At least three independent experiments were performed for each irradiated cell type and the errors were evaluated as standard deviation. Experimental data were fitted with the linear quadratic model (LQ) given by the formula:

$$SF = e^{-(\alpha D + \beta D^2)}$$

where SF is the surviving fraction, D is the dose and α (Gy⁻¹) and β (Gy⁻²) are the radiosensitivity parameters.

Cell-based assays

Methods for DAPI staining, for gene expression analysis on TaqMan Low-Density Array and by real-time RT-PCR as well as for ELISA assays are described in the supplementary Materials and Methods.

RESULTS

Relative biological effectiveness of alpha particles and radiosensitivity parameters for A549 cells and EC

Conventional clonogenic survival assays were performed on both cell types eleven days after irradiation in order to evaluate the relative biological effectiveness (RBE) of alpha particles. Figure 1 shows the dose responses of A549 cells and EC to X-rays and high-LET alpha particles. X-rays (250 kV) were used as reference radiation to calculate RBE. We obtained a characteristic shape of sparsely ionizing radiation as described by Kogel & Joiner [3]. Alpha particle irradiations from doses ranging from 0.2 Gy to 2 Gy doses were performed on A549 cells and EC to obtain the survival curves. As expected for high-LET irradiations, the experimental data could be fitted with a linear model. The RBE of alpha particles was estimated at 5.5 and 4.6 for 10% survival of A549 cells and EC respectively. For A549 cells, the average values for α and β parameters were respectively $0.332 \pm 0.045 \text{ Gy}^{-1}$ and $0.018 \pm 0.005 \text{ Gy}^{-2}$ for X-rays. The radiosensitivity parameters α and β for X-rays for EC were estimated to $0.19 \pm 0.07 \text{ Gy}^{-1}$ and $0.039 \pm 0.024 \text{ Gy}^{-2}$ respectively and the α parameter was equal to $1.90 \pm 0.26 \text{ Gy}^{-1}$ for alpha particles. No significant difference on clonogenic survival curves was observed between the two cell types for each type of irradiation.

Mitotic catastrophe as the main type of cell death induced in A549 cells and EC after X-ray and alpha particle irradiations

Mitotic catastrophe is considered as the major type of cell death induced by ionizing radiation in most non-hematopoietic tumour cells [10]. Morphological alterations of the nucleus such as multinucleation and micronucleation can be observed due to problems in cytokinesis and chromosomes segregation, which will lead to formation of multinucleated giant cells with several micronuclei [11]. As positive controls of apoptosis and mitotic catastrophe, A549 cells and EC were treated with etoposide or paclitaxel respectively and nuclei were labelled with DAPI (Fig. 2A). Etoposide induced the formation of condensed nuclei, a typical feature of cells undergoing apoptosis. Paclitaxel treatment induced the formation of micronuclei and giant multinucleated cells (Fig. 2A). Twenty-four hours after the radiation exposure with X-rays or alpha particles, micronuclei were formed in both cell types while giant multinucleated cells appeared later, at 48 and 72 hours after irradiation (Fig. 2B). Quantification is presented in supplementary figure 1. The percentage of cells undergoing catastrophic mitosis increased with time after irradiation. A correlation was obtained between the percentage of cells undergoing catastrophic mitosis 24h post-irradiation and their survival fraction. This is of interest since the micronucleus frequency has been shown to be a reliable marker of prognostic [12]. As we did not observe any condensed nuclei characteristic of apoptosis, we thus concluded that catastrophic mitosis was the main type of cell death induced after X-ray and alpha particle irradiations in both cell types.

Effects of X-ray and alpha particle irradiations on gene expression in A549 cells and EC

Several studies have shown that irradiated tumour cells secrete proangiogenic factors like growth factors and cytokines that stimulate endothelial cell survival and migration. This interplay between tumour cells and endothelial cells induced by ionizing radiation may increase tumour radioresistance [13]. Thus, we investigated the effects of X-rays and alpha particles on gene expression in A549 cells and EC. Gene expression analysis was performed using Taqman low-density array on both cell types 2.5h and 24h after X-ray or alpha particle

exposure. The complete dataset for A549 cells and EC are presented in supplementary Tables 1 and 2 respectively. Of these, 6 genes were selected for their profile (supplementary figure 2): *CCL2* (or MCP-1, monocyte chemotactic protein-1), *IL1B* (interleukin-1beta), *CSF1* (colony stimulating factor-1), *CXCL10* (or IP-10, interferon inducible protein-10), *IL8* (interleukin-8) and *FAS* (CD95). After X-ray exposure of A549 cells, overexpression of these 6 genes was observed. The overexpression was more pronounced 24h after exposure than after 2.5h for *CCL2*, *CSF1*, *CXCL10* and *IL8*. On the other hand, for endothelial cells, the induction of *CCL2*, *IL1B*, *IL8* and *FAS* was higher 2.5h after the irradiation than after 24h. In order to compare cellular response after both types of irradiation, we have to take into account the RBE of alpha particle. As the RBE for A549 cells and EC was evaluated to 5.5 and 4.6 respectively, we had to compare a dose of 2 Gy for alpha particle with a dose of 10 Gy for X-rays. At biologically equivalent doses, we observed higher gene responses after alpha particle irradiation compare to X-rays in A549 cells but not in endothelial cells (supplementary figure 2). In order to confirm the changes in gene expression observed with TLDA, qRT-PCR assays were performed (Fig. 3). In A549 cells, all the up-regulations observed with TLDA were validated by qRT-PCR except for *CSF1* after alpha particle exposure. We found a good correlation between change folds obtained by TLDA and qRT-PCR. In order to extend these observations, the expression of these 6 genes was also assayed in two other cancer cell lines, HeLa and MCF-7 cells. A similar trend than for A549 cells was observed in both cell types after X-ray irradiation but that did not reach statistical significance except for *FAS* and *CCL2*. On the other hand, alpha particle irradiation did increase the expression of these 6 genes in both cell types, as it was observed for A549 cells (supplementary figure 3). For EC, we confirmed the overexpressions observed by TLDA after X-ray exposure but the increase in gene expression did not reach statistical significance after alpha particle irradiation except for *FAS*.

We also investigated the effects of X-ray and alpha particle irradiations on the gene expression of two well-known angiogenic growth factors: fibroblast growth factor 2 (*FGF2*) and platelet-derived growth factor beta (*PDGFB*). *FGF2* overexpression was observed in A549 cells after both types of irradiation while the expression of *PDGFB* did not change (Fig. 4A). An increase in the expression of *FGF2* but not of *PDGFB* was also observed in MCF-7 cells exposed to X-ray or alpha particle irradiation (supplementary figure 4). On the other hand, no change in *FGF2* gene expression was observed in EC while *PDGFB* was overexpressed after both types of irradiation although the increase in gene expression did not reach statistical significance (Fig. 4B).

Effects of X-ray and alpha particle irradiations on IL-8 and CCL2 secretion by A549 cells and EC

In order to investigate whether the variations in cytokine gene expression translate into actual change in their secretion, ELISA assays were performed for CCL2/MCP-1 and IL-8. Results show that X-ray and alpha particle irradiation of A549 cells significantly increased the secretion of the two cytokines while for endothelial cells, this increase was only observed for CCL2 after X-ray exposure (Fig. 5).

DISCUSSION

The importance of tumour vasculature in the tumour response to radiotherapy was already highlighted by Garcia-Barros et al.. In mice models bearing fibrosarcoma or melanoma, they demonstrated that apoptosis resistance of the tumour vasculature led to an enhanced tumour growth and a decrease in radiotherapy efficacy [14]. By secreting cytokines and growth factors like vascular endothelial growth factor (VEGF) and FGF2, tumour cells could decrease the radiation-induced apoptotic response of their own vasculature [13]. Thus, targeting the interplay between tumour and endothelial cells seems to be a good strategy to

enhance the therapeutic efficacy of ionizing radiation. In this context, we investigated the effect of conventional X-ray and alpha particle irradiations on a lung tumour cell line and endothelial cells in terms of cell survival, cell death and gene expression changes.

Using clonogenic assays, RBE of alpha particles relative to X-rays was determined to be 5.5 and 4.6 for 10% survival for A549 cells and EC respectively. As expected, we demonstrated that high-LET alpha particles were more effective than X-rays. This is consistent with the RBE value of 6.3 for alpha particles relative to gamma irradiation obtained by Beaton et al. at 10% survival for A549 cells [15]. No significant difference on clonogenic survival between A549 cells and EC was observed. This similar radiation response could be explained by the origin of EC used (EAhy926) which are derived from fusion of human umbilical vein endothelial cells (HUVEC) with A549 cells.

Micronucleation was observed 24 hours after irradiation while later, at 48 and 72 hours, multinucleation and giant cells with several micronuclei were observed. These morphological alterations of the nucleus are characteristic of mitotic catastrophe. We thus concluded that mitotic catastrophe was the main type of cell death induced after both types of irradiation. These results are in agreement with the literature given that mitotic catastrophe is now considered as the major cell death mechanism induced by ionizing radiation in solid tumours and in most non-hematopoietic tumour cells [10, 11].

Four well-known pro-inflammatory cytokines were overexpressed after alpha and X-ray irradiations in A549 cells: *CCL2*, *CSF1*, *IL8* and *IL1b*. *CCL2*, also known as MCP-1, promotes inflammation at the tumour site by recruiting monocytes, lymphocytes and natural killer cells. In addition, *CCL2* was reported as a pro-angiogenic chemokine that could induce endothelial cell sprouting and migration of HUVEC and human dermal microvascular

endothelial cells. It has also been shown that CCL2 enhanced tumour growth and metastasis in SCID mice bearing human breast carcinoma cells [16]. A549 cells were also reported to produce high amounts of CCL2 and IL-8 compared with nonmalignant bronchi epithelial cell line [17]. We did observed an increase in the secretion of these two cytokines by A549 cells after X-ray and alpha particle irradiation. The IL-8 pathway was reviewed to have a wide impact in cancer progression because of its pro-angiogenic, pro-inflammatory and pro-survival properties. CSF-1 secretion by tumour cells is implicated in the recruitment of tumour-associated macrophages which can actively promote inflammation, angiogenesis and metastasis [18]. Besides, a high serum level of CSF-1 in patients with NSCLC is a predictor of poor survival [19]. In this context, CCL2, CSF-1, IL-8 and IL-1b represent potential signaling pathways that could be disrupted to increase cancer therapy effectiveness. Because of its pro-angiogenic and radioprotective properties on EC, FGF2 seems to be a good target too [20]. As we observed an upregulation of *FGF2* mRNA level after X-ray and alpha particle irradiations in A549 but also in MCF-7 cells, it could be an interesting to combine FGF2 inhibitor with ionizing radiation.

On the other hand, X-ray and alpha particle irradiations induced the overexpression of CXCL10, an angiostatic chemokine that has been shown to inhibit HUVEC and human lung microvascular endothelial cells proliferation [21]. These anti-angiogenic and antitumour effects make CXCL10 an interesting therapeutic candidate for cancer treatment.

The results presented in this work suggest that EC exposed to ionizing radiation could promote tumour cell growth and radioresistance by secreting pro-inflammatory chemokines and growth factors. Indeed, *CCL2*, *IL8* and *IL1b* were overexpressed after X-ray exposure. The secretion of CCL2 was also enhanced. *CSF1* and *PDGFB* mRNA levels were also upregulated after X-ray and alpha particle irradiations in EC but it did not reach statistical significance either. PDGF-B secreted by HUVEC has been described to stimulate A549 cell

migration [22]. The authors suggested that EC could modulate A549 cell behavior via paracrine signaling. They proposed the existence of intensive signaling loops involving tumour and stromal cells and recommended to disrupt this signaling crosstalk to improve therapeutic response. Molecular targeting in combination with radiotherapy is indeed a promising approach [23].

This potential cross-talk between tumour cells and EC can lead to a reciprocal protection against ionizing radiation and impaired radiotherapy effectiveness. This discovery of new interplay pathways could be targeted during radiotherapy in order to enhance its effectiveness.

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Conflict of Interest Statement

The authors declare no conflict of interest.

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Fig. 1: Survival curves of A549 cells and EC exposed to X-rays or alpha particles. Survival fraction was calculated using conventional clonogenic assay. The LQ model was applied to experimental data (straight lines). Results are presented as means, the error bars represent standard deviation (2 or 3 independent experiments with $n = 3$).

Fig. 2: Nuclear morphology after drug treatment or irradiation. Cell nuclei were labelled with DAPI and pictures were taken using a non-confocal fluorescent microscope (40x magnification). (A) Cells were incubated with etoposide (50 μ M) for 24h or paclitaxel (20 μ M) for 48h. Controls (CTL) are untreated cells. Representative apoptotic cells with chromatin condensation are pointed with white arrows. Paclitaxel treatment induced the formation of multinucleated cells and micronuclei (red arrows). (B) Cells were stained with DAPI at 24, 48 and 72 hours after irradiation with either 5 Gy X-rays or 1 Gy alpha particles. Yellow arrows: micronuclei ; green arrows: multinucleated cells.

Fig. 3: Validation by qRT-PCR of the effects of X-ray (10 Gy) and alpha particle (2 Gy) irradiations on the expression of *CCL2*, *CSF1*, *IL8*, *IL1B*, *CXCL10* and *FAS*. Total RNA was extracted 24h after irradiation for A549 cells (A) and 2.5h after irradiation for EC (B). Results are presented in induction fold as means $\pm$ 1 S.D. ($n = 3$) by comparison with non-irradiated cells (CTL). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ vs corresponding CTL; ns, nonsignificant.

Fig. 4: Effects of X-ray (10 Gy) and alpha particle (2 Gy) irradiations on the expression of *FGF2* and *PDGFB*. Total RNA was extracted 24h after irradiation for A549 cells (A) and 2.5h after irradiation for EC (B). Results are presented in induction fold as means $\pm$ 1 S.D. (n

= 3) by comparison with non-irradiated cells (CTL). *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$ vs corresponding CTL; ns, nonsignificant.

Fig. 5: Effects of X-ray (10 Gy) and alpha particle (2 Gy) irradiations on the secretion of CCL2 and IL-8. Culture medium was recovered 24h after irradiation for A549 cells (A) and EC (B). CCL2- and IL-8 was then assayed using specific ELISAs. Results are presented in pg/ μ g of total proteins, as means $\pm$ 1 S.D. (n = 3). *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$ vs corresponding CTL; ns, nonsignificant.

Supplementary data:

Fig. 1: Quantification of mitotic catastrophe. Cell nuclei were labelled with DAPI and pictures were taken using a non-confocal fluorescent microscope (40x magnification) (A) Quantification of cells undergoing mitotic catastrophe 24h, 48h and 72h after irradiation of A549 cells and of E with X-rays or alpha particles. At least 100 cells were counted to evaluate the percentage of cells undergoing mitotic catastrophe. (B) Correlation between the percentage of cells undergoing mitotic catastrophe 24h post-irradiation and their survival fraction.

Fig. 2: Gene expression profiles for *CCL2*, *CSF1*, *IL8*, *IL1B*, *CXCL10* and *FAS* after X-ray or α -particle irradiation. Total RNA was extracted 2.5h and 24h after radiation exposure and retrotranscribed before being amplified in TaqMan low-density array. Results are presented in induction fold by comparison with non-irradiated cells.

Table 1: Gene expression profiles in A549 cells as a function of radiation dose of X-rays and α -particles. Total RNA was extracted 2.5h and 24h after radiation exposure and retrotranscribed before being hybridized onto TaqMan low-density array human immune as described in Materials and Methods. Results are presented in induction fold by comparison with the reference condition, un-irradiated cells. NE: not expressed i.e. Ct value > 35 or undetermined.

Table 2: Gene expression profiles in EC as a function of radiation dose of X-rays and α -particles. Total RNA was extracted 2.5h and 24h after radiation exposure and retrotranscribed before being hybridized onto TaqMan low-density array human immune as described in Materials and Methods. Results are presented in induction fold by comparison with the reference condition, un-irradiated cells. NE: not expressed i.e. Ct value > 35 or undetermined.

Fig. 3: Validation by qRT-PCR of the effects of X-ray (10 Gy) and alpha particle (2 Gy) irradiations on the expression of *CCL2*, *CSF1*, *IL8*, *IL1B*, *CXCL10* and *FAS*. Total RNA was extracted 24h after irradiation for HeLa (A) and MCF-7 cells (B). Results are presented in induction fold as means $\pm$ 1 S.D. (n = 3) by comparison with non-irradiated cells (CTL). *, p<0.05; **, p<0.01; ***, p<0.001 vs corresponding CTL; ns, nonsignificant.

Fig. 4: Effects of X-ray (10 Gy) and alpha particle (2 Gy) irradiations on the expression of *FGF2* and *PDGFB*. Total RNA was extracted 24h after irradiation for HeLa (A) and MCF-7 cells (B). Results are presented in induction fold as means $\pm$ 1 S.D. (n = 3) by comparison with non-irradiated cells (CTL). *, p<0.05; **, p<0.01; ***, p<0.001 vs corresponding CTL; ns, nonsignificant.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

A

B

Supplementary figure 1

A

	Dose	SF (%)	Cells undergoing mitotic catastrophe (%)		
			24h	48h	72h
A549 cells	5 Gy X-rays	12,1	9	32	58
	1 Gy α-particles	9,4	7	51	45
Endothelial cells	5 Gy X-rays	14,6	18	43	44
	1 Gy α-particles	14,9	14	43	42

B

Supplementary figure 2

A549 cells

Endothelial cells

CXCL10
not expressed in EC

Supplementary figure 3

Supplementary figure 4

Supplementary Materials and Methods

DAPI staining

Twenty-four hours before X-ray irradiation, A549 cells and EC were seeded on glass cover slides in 24-wells culture plate at 30,000 cells/well and 50,000 cells/well respectively. At indicated time points after irradiation, cells were fixed for 15 min in 4% paraformaldehyde (Merck). After three PBS washes, cells were permeabilized in PBS + 0.2% triton X100 (Merck) for 5 minutes at room temperature. Cells were stained with 4',6-diamidino-2-phenylindole (DAPI; Sigma, 100 ng/ml) for 15 min in dark at 37°C. After one PBS wash, coverslips were mounted in Mowiol (Sigma) and observed with a non-confocal fluorescent microscope.

At indicated time points after alpha particle irradiation, cells were trypsinized and split into two wells containing glass cover slides in 24-wells culture plates. At indicated time points, cells were fixed. DAPI staining was performed as described above. At least 100 cells were counted to estimate the percentage of cells undergoing mitotic catastrophe. As positive controls of apoptosis and mitotic catastrophe, both cell types were incubated respectively with etoposide (Sigma) at 50 μ M during 24h in medium or paclitaxel (Invitrogen) at 20 μ M during 48h in medium containing 10% serum.

Gene expression analysis on TaqMan Low-Density Array

Total RNA was extracted using the Total RNAgent extraction kit (Promega). The "High Capacity cDNA Archive" kit from Applied Biosystems was used to reverse transcribed 1 μ g total RNA. PCR amplifications on TLDA Human Immune were performed in a 7900HT Fast Real-Time PCR system. Relative fold-inductions were calculated by the comparative cycle threshold method ($2^{-\Delta\Delta C_t}$). To determine the most appropriate endogenous control, we used

Genorm on the 6 housekeeping genes. The housekeeping gene used for normalization was GAPDH.

Quantitative real-time PCR

Total RNA was extracted with the RNeasy kit (Qiagen). 1 µg total RNA was reverse transcribed into cDNA using SuperScript II Reverse Transcriptase (Invitrogen). Amplification reaction assays contained SYBR Green PCR Mastermix (Applied Biosystems) and primers at the optimal concentrations. The relative fold-induction was calculated by the cycle threshold method and the housekeeping gene used for normalization was GAPDH. Experiments were performed in triplicates and statistical analyses were performed with SigmaStat software (Jandel Scientific). Data are presented as means $\pm$ 1 S.D. and were evaluated by Student's t test.

ELISA

IL-8 and CCL2 secretion in the culture medium was assessed using Quantikine ELISA (R & D Systems) according to the manufacturer's protocol. Experiments were performed in triplicates and statistical analyses were performed with SigmaStat software (Jandel Scientific). Results are presented in pg/µg total proteins assessed by the Folin method, as means $\pm$ 1 S.D. and were evaluated by Student's t test.