



Research paper

Secondary cytotoxicity mediated by alveolar macrophages: A contribution to the total efficacy of nanoparticles in lung cancer therapy?

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ABSTRACT

Local treatment of lung cancer using inhalable nanoparticles (NPs) is an emerging and promising treatment option. The aim of this study was to investigate the activation of alveolar macrophages by poly (iso-butyl cyanoacrylate) (BIPCA) NPs and the consequences of this activation on H460 lung cancer cells. A methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay was used to determine the primary cytotoxicity, that is, the immediate and direct cytotoxicity of doxorubicin (DOX)-loaded NPs on both cell lines. Macrophages were then treated using EC₅₀ concentrations of different treatments and co-cultured in a two-compartment system with H460 lung cancer cells. These treatments included DOX solution, blank NPs, and DOX-loaded NPs.

The results showed that alveolar macrophages exposed to blank or DOX-loaded NPs showed cytotoxicity against cancer cells after 8 and 24 h; this behavior was not expressed by naïve macrophages or macrophages treated with DOX solution.

Sample analysis indicated that macrophages have the ability to release back fragments of NPs that were previously phagocytized. Further investigations showed that NPs can induce an increase in the excretion of Th1 cytokines namely, monocytes chemoattractant protein-1 (MCP-1), macrophages inflammatory protein (MIP-1), tumor necrosis factor α (TNF- α), and interferon gamma (IFN- γ).

The Th1 cytokines released by the alveolar macrophages might explain the significant secondary cytotoxicity effect on H460 cancer cells. Secondary cytotoxicity mediated by macrophages might compliment the direct cytotoxic effect that NPs have on cancer cells.

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1. Introduction

NPs have proven to be promising drug carriers in cancer therapy due to their ability to accumulate in cancer tissues by what is known as an enhanced permeability and retention (EPR) effect [1]. Moreover, NPs are able to modulate and overcome multidrug resistance *in vitro* and are targeted to special types of cells or tissues, passively and/or actively [2]. The main problem encountered when NPs were used as drug carriers was the interference of macrophages of the mononuclear phagocytotic system (MPS) [3]. The high uptake of NPs in the liver, mediated by Kupffer cells, was used to explain the observed improved efficacy [4]. It was hypothesized that Kupffer cells have the ability to phagocytize NPs after *i.v.* administration as

they pass through the liver and then to release them back by exocytosis, in close proximity to carcinoma cells in the liver causing an enhanced cancer cell death at this location [5].

In addition to *i.v.* injections, several routes of administration for NPs are being examined, such as dermal, ocular, and pulmonary delivery [6–8]. Pulmonary delivery is becoming an important route of drug administration [9]. This is supported by the lung's unique characteristics such as large surface area, thin epithelial layer, high vascularization, and less first-pass metabolism impact. The lungs, as a part of the MPS, are extensively rich in alveolar macrophages [10]. Therefore, macrophages are expected to have a substantial effect on the fate of inhaled NPs and the efficacy of any treatment using inhalable NPs as a delivery system [11]. As previously shown, the fate of inhalable NPs is mainly determined by their size and surface characteristics [6]. One issue with NP pulmonary delivery is that their size is not suitable for deep lung deposition. In fact, a carrier system such as lactose microparticles is required for deep lung delivery. The carrier particles should meet special requirements

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such as an appropriate mass median aerodynamic diameter (MMAD) and a suitable fine particle fraction (FPF) [9,12,13]. In general, a portion of the inhaled NPs evades macrophages and translocates out of the alveolar spaces to other lung tissues and to the general circulation [14]. Another fraction of the inhaled NPs will be cleared out by the alveolar macrophages, similar to other foreign objects brought into the lungs during breathing [15]. Consequently, the effectiveness of any anticancer therapy using inhalable NPs will be affected by the extent to which inhaled NPs are cleared by macrophages. The presence of alveolar macrophages limits the chance of NPs to reach cancer cells and decreases their efficacy as anticancer agents. However, alveolar macrophages might act in the lung similarly to what was suggested for Kupffer cells in the liver. Generally, alveolar macrophages are in continuous movement, migrating between the lungs' different tissues and the lymphatic system [16]. In the case of lung tumor, alveolar macrophages also infiltrate the tumor tissue and become tumor-associated macrophages (TAM) [17]. Macrophages activated by NPs might interact with cancer cells differently than naïve macrophages in favor of the cancer rejection. This study was designed to mimic, *in vitro*, a lung cancer tumor xenograft animal model in which murine alveolar macrophages are in contact with human lung cancer cells. Moreover, to investigate other pathways that NPs might affect cancer cells other than the direct cytotoxic effect.

2. Materials and methods

2.1. Materials

Dextran, methylthiazolyldiphenyl-tetrazolium bromide (MTT), 1% trypsin–EDTA, and trypan blue were purchased from Sigma (Ontario, Canada). RPMI-1640 medium and cell culture supplements were supplied from Invitrogen (Ontario, Canada). Isobutyl cyanoacrylate monomer (Lot. 02GD9236) was a gift from Loctite Ltd. (Dublin, Ireland). Doxorubicin (DOX) was purchased from Novopharm Ltd. (Ontario, Canada). Human non-small cell lung carcinoma cells (H460) and murine alveolar macrophages (MH-S) cell lines were obtained from American Type Culture Collection (ATCC, Rockville, USA).

2.2. Nanoparticle (NP) preparation and characterization

Poly (isobutyl cyanoacrylate) (PIBCA) NPs were prepared using an emulsion polymerization method described previously [18,19]. Briefly, 100 mg of dextran was added to 10 mL of 0.01 N hydrochloric acid, then 100 μ L of isobutyl cyanoacrylate monomer was added under continuous stirring at 500 rpm. The final NP dispersion was filtered using a 0.8- μ m-nucleopore[®] membrane filter from Whatman (Ontario, Canada) under vacuum. Concentrations of the NP dispersions were determined using a gravimetric method. Then, 1 mL of NP dispersion was placed in a porcelain dish and the sample was heated at 60 °C until it was dry, and the difference in weight was used to calculate the NP dry matrix weight [19]. DOX-loaded NPs were prepared by adding 2.4 mg of DOX in a 2 mg/mL solution 30 min after addition of the monomer. The mixture was stirred continuously for 4 h in the dark. The loading efficiency was determined by calculating the difference between the added amount of DOX and the unbound fraction of DOX. The unwashed NP dispersion was centrifuged at 17,000 rpm for 10 min using a 5415C[®] microcentrifuge from Eppendorf (Hamburg, Germany). The supernatant was separated and analyzed for free DOX. The centrifuged NP pellets were separated and dried, and the weight was used to calculate the loading capacity. The loading efficiency was 80% and the loading capacity was 9.6 mg/100 mg of NPs. These values are in agreement with which was reported previously [20,21]. The loading capacity value was used later to calculate the DOX concentration for the primary

toxicity study. Particle size and zeta potential were measured by photon correlation spectroscopy using a Zetasizer HAS 3000 from Malvern (Worcestershire, UK). The Z-average value was used to express the mean hydrodynamic particle size in nm, and the polydispersity index was used to indicate the width of distribution. The final NP preparations were tested for endotoxin presence using an E-Toxate[™] Kit (Sigma, Canada).

2.3. Cell culture

Murine alveolar macrophages (MH-S) and human non-small cell lung carcinoma cells (H460) were cultured in 25-ml ventilated flasks (Corning, USA) using RPMI-1640 medium supplemented with 0.11% sodium pyruvate, 1% nonessential amino acids, 1 mM HEPES buffer, 0.15% sodium bicarbonate, 100 IU/ml penicillin, 10 μ g/ml streptomycin, and 10% heated inactivated fetal bovine serum. Cells were maintained in a humidified incubator at 37 °C in an atmosphere containing 5% CO₂.

2.4. Primary cytotoxicity and EC₅₀ values of treatments

The primary cytotoxicity of four different treatments, DOX solution, blank NPs, mixture of blank NPs and DOX solution, and DOX-loaded NPs, on both MH-S and H460 cells was assessed using an MTT assay. Briefly, the cells grown in flasks were washed with phosphate-buffered saline (PBS) and trypsinized using 1% trypsin–EDTA. The cells were centrifuged, the supernatant was discarded, and the cells were resuspended in RPMI-1640 complete medium. Approximately 5000 cells were counted using a hemacytometer and seeded in each well of a 96-well plate. The plates were incubated for 24 h at 37 °C under 5% CO₂. After 24 h, the wells were rinsed with PBS, and different treatments were added in serial concentrations. The DOX concentration value was used to indicate the primary cytotoxicity of DOX-loaded NPs and DOX solution. NP concentration, determined based on the dry weight of PIBCA polymer, was used to refer to the primary toxicity of blank NPs. Blank NPs had the same concentration based on dry weight as DOX-loaded NPs for comparison purposes. The treatment was washed off after 1 h, and the cells were rinsed with PBS three times. Then, 100 μ L of 0.5 mg/ml MTT was added to each well. After 2 h, the MTT solution was removed, 100 μ L of isopropanol was added to the plates, and the plates were shaken for 1 h. The color intensity of the wells was measured at 550 nm using a bio-Tek EL 312e microplate reader (Winooski, VT). The EC₅₀ of each treatment was calculated using a linear best-fit line.

2.5. Secondary cytotoxicity mediated by macrophages

A 24-mm transwell[®] co-culture system (Costar Inc., USA) was used to evaluate the ability of murine macrophages to mediate the secondary cytotoxicity of different treatments on H460 cells. Each transwell[®] plate contained 6 wells and 6 inserts, each insert and well were separated by a 0.4- μ m porous membrane. Approximately 1×10^5 H460 cells were implemented into the lower compartment of each well, and plates were incubated for 24 h. Aliquots containing EC₅₀ concentrations of different treatments were added to MH-S cells grown in separate flasks. After 1 h, MH-S cells were washed 3 times with PBS, trypsinized, centrifuged, washed again, counted, and then added to the upper compartment. The following macrophage/cancer cell ratios were tested in the co-culture system: 1:10, 1:5, 1:1, 5:1, and 10:1. No treatment of any kind was added to the upper compartment; only macrophages were seeded in the upper compartment. Naïve macrophages, not previously treated, were seeded in the upper compartment, and results were used as control. The viability of the cancer cells in the lower compartment of each well was tested at 1, 8, and 24 h using the MTT assay previously mentioned.

2.5.1. Sample collection

Using the same co-culture system, macrophages incubated with different treatments were seeded into the upper compartment. Samples were collected from the lower compartment, filled with medium after 1, 8, and 24 h, and analyzed for DOX and changes in the cytokine secreting profile.

2.5.2. HPLC analysis

Each sample was added to 250 μ l of water, vortexed for 30 s, and centrifuged at 17,000 rpm. Aliquots from the supernatant were analyzed by HPLC. The HPLC system consisted of a 851-AS[®] auto sampler (Jasco Co., Tokyo, Japan), a LC-600[®] isocratic pump (Shimadzu Co., Tokyo Japan), and a lichosphere[®]100 RP18e (5 μ m) cartridge (Lichocart, Merck, Germany). Samples were eluted with a mixture of water, acetonitrile, methanol, and phosphoric acid (540:290:170:2) at a flow rate of 1 ml/min. DOX was detected with an FP-920[®] fluorescence detector (Jasco Co., Tokyo, Japan) adjusted to 460-nm excitation wavelength and 550-nm emission wavelength. This analysis detected free DOX in the samples as PIBCA NPs are not water soluble. To analyze the total DOX—free DOX and DOX attached to NP fragments—samples were treated with 250 μ l of acetonitrile and the mixture was vortexed for 2 min to ensure that the cyanoacrylate polymer was totally dissolved before the samples were centrifuged as above, and aliquots of the supernatants were injected into the HPLC system [22]. Appropriate calibration curves were prepared using appropriate serial concentrations of DOX solution. The experiments were repeated three times with two technical duplicates in each experiment.

2.5.3. Cytokine analysis

Changes in cytokine secreting profiles, induced by different treatments, were assessed using a Raybio[®] cytokine antibody array (Ray biotech Inc., USA). Membranes covered with primary antibodies were incubated with the collected samples overnight at 4 °C in a plastic plate included in the kit, then washed using the buffer provided in the kit. After the antibody–cytokine complexes were formed, membranes were incubated with biotinylated secondary antibodies and then with labeled streptavidin. The membranes were exposed to X-ray film for 5 s, and the film was developed. The intensity of the signal of each spot representing a specific cytokine was evaluated using Imaj software. The experiments were repeated three times with two technical duplicates in each experiment. Each membrane contained six control spots located on the left-upper and right-lower of each membrane to confirm that the membranes were treated correctly throughout the different experiments. The average reading of the six control spots was used to normalize the reading in each membrane separately. In order to confirm that the cytokines detected in this experiment were excreted from macrophages and were not an artifact of the cell-culturing medium, a cytokine-free RPMI-1640-conditioned medium was used.

2.6. Statistical analysis

Statistical analysis was performed using a single factor ANOVA test or *T* test, as appropriate, with a 0.05 level of significance.

3. Results

3.1. NP properties

The prepared blank NPs had an average size of 137.22 ± 1.53 nm, the polydispersity index was 0.12, and the zeta potential was -23.5 ± 0.41 mV. No significant changes were observed with DOX-loaded NPs as the average particle size observed was

140 ± 1.98 nm, the polydispersity index was 0.21 and the zeta potential was -21.56 ± 0.32 mV. An E-Toxate[™] test indicated that tested NP preparations were endotoxin free.

3.2. Primary toxicity and EC₅₀

Fig. 1 shows the primary cytotoxicity of different treatments on MH-S and H460 cells. As expected, DOX-loaded NPs exhibited the highest primary cytotoxicity in both cell lines. Moreover, the EC₅₀s of DOX-loaded NPs were similar, 0.24 μ g/ml and 0.26 μ g/ml for MH-S and H460 cells, respectively. Furthermore, the primary cytotoxicity of DOX-loaded NPs in both cell lines was higher than an equal mixture of blank NPs and DOX solution. The effect of the mixture was less than the additive cytotoxicity of the individual components. Therefore, the mixture of blank NPs and DOX solution was excluded from secondary toxicity experiments. Although both cell lines, MH-S and H460, showed almost the same sensitivity toward DOX-loaded NPs, blank NPs showed higher cytotoxicity for macrophages than for lung cancer cells, as indicated in Table 1. DOX solution showed significant cytotoxicity in both cell lines; however, MH-S cells were less sensitive than H460 cells.

3.3. Secondary cytotoxicity of NPs mediated by macrophages

Secondary cytotoxicity was defined in this study as an acquired cytotoxicity exerted by phagocytotic cells on cancerous cells. This special cytotoxicity is described as acquired because it is a result of macrophage exposure to NPs and to the fact that this property was not expressed by naïve macrophages. Alveolar macrophages were proved able to mediate secondary cytotoxicity on H460 cells. The extent of secondary cytotoxicity was dependent on both the type of treatment and the cell ratio used in the two-compartment co-culture system. Low MH-S to H460 cell ratios failed to produce any significant secondary cytotoxicity. The cytotoxicity started to be detectable at a 1:1 cell ratio, but the results were more significant at 5:1 and 10:1 ratios. The last two ratios showed almost the

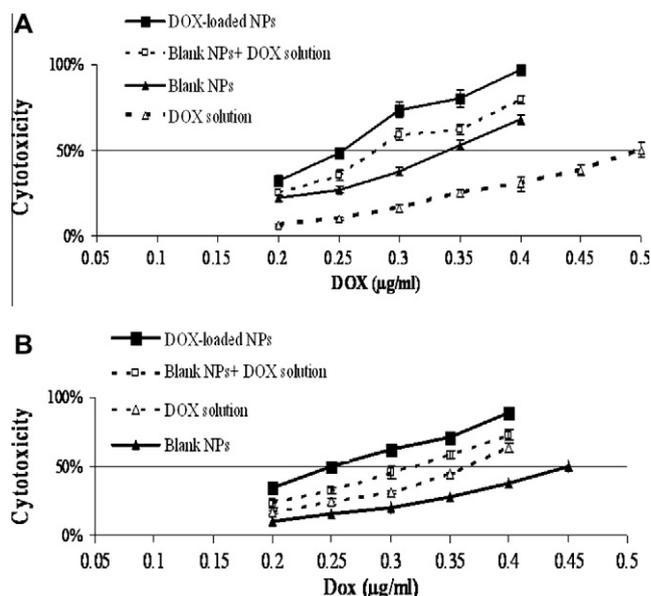


Fig. 1. Primary cytotoxicity of four different treatments: DOX solution, blank NPs, mixture of blank NPs and DOX solution, and DOX-loaded NPs, on MH-S (murine alveolar macrophages) (A) and H460 (human non-small cell lung carcinoma) cells (B). Each data point represents the average of six wells and three separate experiments. The concentration of DOX solution and DOX-loaded NPs was calculated according to the DOX concentration (μ g/ml). NP loading capacity value was used to correlate between NP dry matrix and DOX concentration.

Table 1The EC₅₀ values of different treatment on MH-S cells (macrophages) and H460 cells (lung carcinoma).

	DOX solution	Blank NPs	DOX-loaded NPs
MH-S cells	EC ₅₀ of DOX = 0.52 ± 0.02 µg/ml [*] NP dry matrix (N/A)	Blank NP matrix to exert EC ₅₀ = 3.54 ± 0.12 µg/ml [†] EC ₅₀ of DOX (N/A)	EC ₅₀ of DOX = 0.24 ± 0.01 µg/ml equivalent to NP dry matrix of 2.5 µg/ml
H460 cells	EC ₅₀ of DOX = 0.35 ± 0.01 µg/ml NP dry matrix (N/A)	Blank NP matrix to exert EC ₅₀ = 4.79 ± 0.18 µg/ml EC ₅₀ of DOX (N/A)	EC ₅₀ of DOX = 0.26 ± 0.02 µg/ml equivalent to NP dry matrix of 2.71 µg/ml

^{*} *P* < 0.05.[†] NPs concentration was calculated depending on the value of loading capacity.

same extent of cytotoxicity with a better reproducibility obtained with a 5:1 cell ratio. Therefore, a 5:1 ratio was chosen for further investigation.

Fig. 2 shows the secondary cytotoxicity induced by different treatments and mediated by macrophages at different time points using a MH-S:H460 5:1 cell ratio. As shown, the DOX solution did not induce alveolar macrophages to cause any secondary toxicity at any time point. On the other hand, macrophages treated with blank or DOX-loaded NPs showed an acquired toxicity against cancer cells after 8 and 24 h. Naïve macrophages did not exert any secondary cytotoxicity against cancer cells and the viability of cancer cells in the lower compartment was comparable with results obtained when no macrophages were added to the upper compartment.

3.4. DOX released from macrophages

To further investigate the mechanistic causes of the observed secondary cytotoxicity, samples were collected from the lower compartment and analyzed for both free and total DOX. Total DOX consisted of the free DOX existing in the sample and the fraction of DOX still attached to NP fragments. When macrophages were incubated with DOX solution and seeded in the upper compartment, no DOX was detected in the sample collected from the lower compartment, whereas free and NP-attached DOX was detected in the samples when macrophages were previously treated with DOX-loaded NPs. This indicated that NP fragments were released from macrophages after DOX-loaded NPs were phagocytized. Fig. 3 shows the amount of free and total DOX detected in the samples collected from the lower compartment after treating macrophages with DOX-loaded NPs.

Almost equal amounts of free DOX were detected at all time points with no significant increase after 24 h, whereas no attached

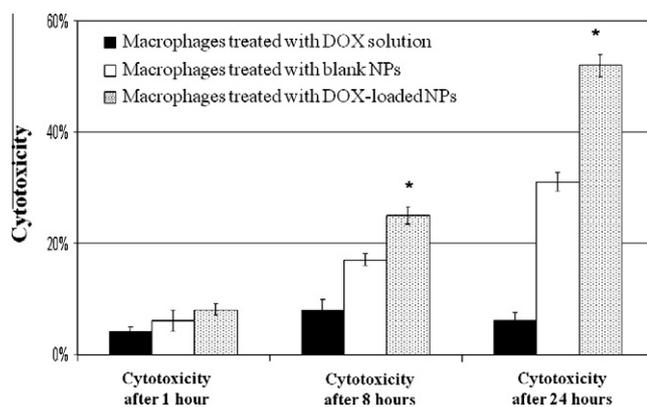


Fig. 2. The secondary cytotoxicity mediated by murine alveolar macrophages (MH-S) on human non-small cell lung carcinoma cells (H460) at a 5:1 MH-S:H460 cell ratio. Macrophages were incubated with EC₅₀ aliquots of different treatments for 1 h before being added to the upper compartment of the transwell® co-culture system. Each value represents the average of two wells and three independent experiments (^{*} *P* < 0.05).

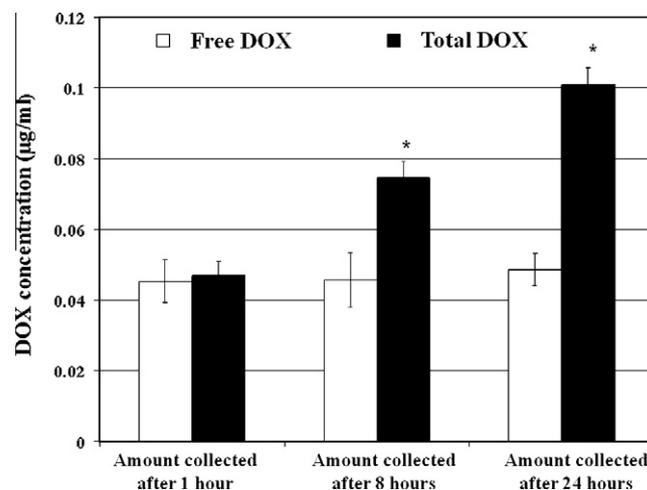


Fig. 3. The amount of free and total DOX detected in samples collected from macrophages treated with DOX-loaded NPs at different time points. Each value represents the average of two technical repetitions of three separate experiments (^{*} *P* < 0.05).

DOX was observed in samples collected after 1 h as the total DOX was equal to the free DOX detected. On the other hand, a significant increase in total DOX was detected after 8 and 24 h (Fig. 3).

3.5. Cytokine secreting profile

Changes in the cytokine secreting profile of macrophages were investigated after macrophages were exposed to different treatments. The type and the amount of cytokines were closely related to both the type of treatment and the sampling time point. The results showed a significant increase in the secretion of Th1-type cytokines which are known to induce acute inflammation and cell-mediated immune response. The increase in cytokine secretion was observed after 8 and 24 h and was limited to macrophages treated with blank and DOX-loaded NPs. Monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1 (MIP-1), tumor necrosis factor α (TNF- α), and interferon gamma (IFN- γ) all belong to the group of Th1 cytokines (Fig. 4). MCP-1, followed by MIP-1, was the main cytokine triggered by NPs; the increase was about threefold compared with control samples collected at 24 h (Fig. 5).

No changes in cytokine secreting profiles were observed over time in naïve macrophages used as control, or in macrophages previously incubated with DOX solution. No significant difference in cytokine secreting profile was observed between macrophages treated with blank or DOX-loaded NPs.

4. Discussion

BALB/C nude mice are a commonly used model for establishing the antitumor efficacy of newly developed anticancer molecules or

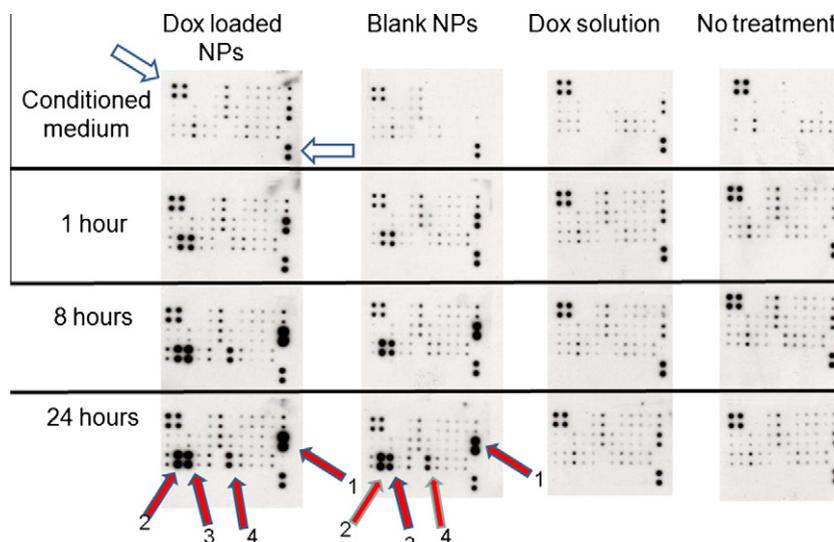


Fig. 4. Blots of the cytokines antibody-array membranes of naïve macrophages (no treatment) and macrophages treated with three different treatments at different time points. Filled arrows point to four different cytokines: MCP-1 (1), MIP-1 (2), TNF- α (3), and INF- γ (4). Empty arrows point to the control spots in one of the membranes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

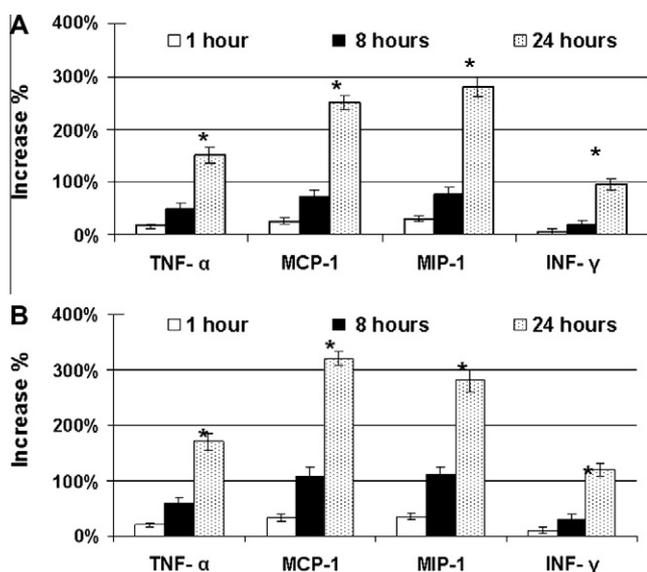


Fig. 5. Increase in cytokine secretion induced by blank NPs (A) and DOX-loaded NPs (B) at different time points. Values are expressed relative to the negative control (conditioned medium). Each value represents the average of two technical repetitions of three separate experiments ($P < 0.05$).

delivery systems. We previously reported that inhalable NPs are well tolerated in this species [23]. The aim of this study was to investigate whether other anticancer mechanisms exist besides the primary cytotoxic effect of NPs on cancer cells.

In this study, DOX-loaded NPs showed superiority in reducing the viability of both macrophages and cancer cells as a result of primary cytotoxicity. Although MH-S and H460 cell lines reacted in a similar way to DOX-loaded NPs, they showed different sensitivities to blank NPs and DOX solution. Alveolar macrophages were more affected by blank NPs when compared to lung carcinoma cells. This can be explained by the difference in the phagocytotic functionality between the cell lines [24]. Alveolar macrophages are expected to have a higher ability to internalize blank NPs; thus they receive a higher amount of cytotoxic poly (isobutyl cyanoacrylate) polymer [25]. The results also showed that the cytotoxicity of the

individual components in a mixture of blank NPs and DOX solution was neither synergistic nor additive. This can be explained by the fact that DOX can decrease the phagocytosis of macrophages by about 10% [26]. Therefore, this mixture was excluded from further investigation. The primary cytotoxicity of different treatments was assessed after a short period of incubation (1 h). Using a short incubation time allowed us to measure the primary cytotoxicity of different treatments before it caused an inhibitory effect; 1 h was sufficient for macrophages to complete the phagocytosis process. Lower concentrations with longer incubation times might show different results [19].

To assess secondary cytotoxicity, macrophages were incubated with aliquots containing EC_{50} concentrations of different treatments for 1 h, which is a sufficient time for phagocytosis to be completed [27]. The secondary cytotoxicity detected in this study showed two main characteristics. First, it was exclusive to NPs (blank or DOX-loaded), as DOX solution did not induce secondary cytotoxicity at any time point. Second, unlike primary toxicity, secondary toxicity was not instantaneous, as no significant increase in secondary toxicity was detected after 1 h (Fig. 2). The fact that blank NPs but not DOX solution showed a high ability to trigger secondary cytotoxicity confirms that the act of phagocytosis triggers intracellular changes causing secondary cytotoxicity. A high macrophage to cancer cell ratio was required to achieve a significant increase in secondary cytotoxicity; the best results were observed with a 5:1 cell ratio. The need for a high cell ratio can be explained by the lack of direct contact between macrophages and cells due to the volume of the medium used in the co-culture system. The fact that macrophages are in close approximation to cancer cells at the tumor site might augment the effect of secondary toxicity seen *in vitro* and enhance its significance. Future *in vivo* studies are needed to validate this assumption. However, other studies have shown that non-small cell lung cancer tumors are usually infiltrated with a large number of macrophages, up to 1823 cells/mm³ [28]. Furthermore, lung cancer cells eventually spread to the regional lymph node which is considered to be an important factor in lung cancer staging [29]. Videira et al. reported a significant lymphatic NP uptake after inhalation; this was explained by the ability of macrophages to act as NP reservoirs linking the alveolar spaces to lymph nodes [30]. Therefore, lymph nodes can be considered potential locations for secondary cytotoxicity.

In order to investigate the mechanism of secondary cytotoxicity, samples were collected for further analysis from the lower compartment of the co-culture system after 1, 8, and 24 h. The ability of macrophages to release back the DOX was treatment dependent. Macrophages treated with DOX solution did not release back any DOX; however, macrophages treated with DOX-loaded NPs released back a fraction of the DOX applied to the NPs. This is explained by the macrophage cellular reaction for different treatments. When in contact with macrophages, DOX solution diffuses passively through the cell membrane, whereas DOX-loaded NPs are internalized actively by phagocytosis [31]. Exocytosis, a natural consequence of phagocytosis, could also be used to explain this observation [32].

In this study, we differentiated between two types of DOX released from the macrophages treated with NPs: free DOX and NP-attached DOX. PIBCA NPs are water insoluble, yet they are biodegradable. The release of the encapsulated drug depends mainly on NP degradation by surface erosion and/or solubilization [33]. Therefore, in order to measure the total DOX in the collected samples (free and bound), NPs must be totally dissolved. Acetonitrile was used to dissolve NPs in the collected samples [22]. In the samples collected after 1 h, the total DOX was not significantly different from free DOX, suggesting that the amount of DOX released from NPs was insignificant. Conversely, a significant increase in total DOX was detected after 8 and 24 h. This increase is attributed to the DOX attached to NP fragments, but not to the free DOX as no significant increase was detected in the free DOX at different time points. The significant difference between total and free DOX after 8 and 24 h, but not after 1 h, suggests that additional DOX, attached to NP fragments, was released from macrophages. This process was not instantaneous; there was a delay before a significant increase in total DOX concentration was observed. This can be explained by the time required to complete the exocytosis process [34]. Even though surface-associated NPs may contribute to the total DOX detected in the samples, the ratio of surface-associated NPs to phagocytized NPs in the phagocytotic cells did not exceed 1:4 [35]. Moreover, using 1% trypsin-EDTA solution to de-attach macrophages may have also decreased the number of NPs associated with macrophage surfaces [36]. Even though alveolar macrophages were able to release fragments of NPs, the concentrations of total DOX detected at different time points were not itself enough to explain the secondary toxicity. It is well documented that after phagocytosis, macrophages undergo different intra- and extracellular changes as defensive measures [37]. Changes in the macrophage cytokine secreting pattern are one of these measures [38]. Cytokines compromise numerous numbers of mediators that profoundly affect biological aspects of a tumor such as proliferation, infiltration, and cell-cell interactions [39]. Some cytokines have direct antitumor effects, and others act indirectly by recruiting and activating cytotoxic cells [40]. Moreover, cytokines such as TNF- α , IL-2, and IFN- γ have been tested *in vitro* and *in vivo* as agents for cancer therapy [41].

Our results showed a significant increase in Th1 cytokines after 8 and 24 h, but not after 1 h, in macrophages exposed to NPs compared with naïve macrophages and macrophages treated with DOX solution. Changes in the cytokine secreting profiles were limited to macrophages treated with NPs, either blank or DOX-loaded, and these changes were very similar (Figs. 4 and 5). This indicates that these changes were not related to DOX but were related to the presence of NPs, and consequently to phagocytosis. Cytokines induced by NPs in this study were MCP-1, MIP-1, TNF- α , and IFN- γ , listed in order of the extent of induction. MCP-1 and MIP-1 belong to the chemokines superfamily. Chemokines possess chemotactic activity for immune and inflammatory cells [42]. MCP-1 activates the tumoricidal activity of monocytes and macrophages *in vitro* and *in vivo* [43]. Besides its ability to activate and

recruit immune cells, MCP-1 induces macrophages to release nitric oxide [44,45]. Nitric oxide mediates DNA damage and demonstrates anticancer activity [45]. MIP-1 is mainly excreted by macrophages after phagocytosis, and its role in the inflammation process has been investigated in diseases such as asthma, arthritis, and multiple sclerosis [46]. Derivatives of MIP-1 were effective in inhibiting of tumor growth after local radiation [47]. In the same context, TNF- α has a direct cytotoxic effect on cancer cells and promotes apoptotic response to some anticancer agents [48]. IFN- γ is an immunity modulator and is synergistic with the TNF- α cytolytic effect on cancer cells [49]. Due to the study design, these results may not reflect an instantaneous macrophage secretion of cytokines after NP exposure. However, our aim was to focus on the later consequences of macrophage activation by NPs. As shown in Figs. 4 and 5, the increase in cytokine secretion was significant over time, but not after 1 h. Due to the time required for macrophage migration and infiltration [50], the therapeutic effect of secondary cytotoxicity might be delayed.

Endotoxins are known to stimulate macrophages and induce the excretion of pro-inflammatory factors such as cytokines. However, it has been shown that endotoxin contamination is mainly associated with high levels of IL-1, IL-6, and TNF- α [51]. The NP preparations used in this study were endotoxin free. Our study showed that MCP-1 and MIP-1 were the main chemokines induced by NPs followed by TNF- α and IFN- γ ; IL-1 and IL-6 were not induced significantly by NPs in this study.

Cancer builds its own microenvironment surrounded by chronic inflammation [52]. Chronic inflammation is generally associated with antibody-mediated humoral immune response, which is mainly mediated by Th2 cytokines [53]. Th1 cytokines promote acute inflammation and cell-mediated immune responses that aid cancer rejection [54]. Therefore, Th1 cytokines are associated with higher survival, whereas Th2 cytokines help build a microenvironment that makes a tumor resistant to anticancer agents [55]. Because immune responses are controlled mainly by pro-inflammatory secondary toxicity, aggressive behavior of alveolar macrophages induced by NPs might favor cell immunity and help in cancer rejection. Thus, inhalable NPs might be effective in lung cancer treatment. Induction of other cytokines was consistent among all treatment groups, including the naïve macrophages (no treatment received); their concentrations were low and irrelevant to the type of the treatment.

We used H460 and MH-S cell lines to mimic the mouse model that has become an important tool to establish *in vivo* antitumor activity of new anticancer agents and/or delivery systems. In this model, many cell-cell interactions during tumor development and recession are common to both human and murine species [56]. The interspecies activity of cytokines such as TNF- α , MCP-1, and IL-6 has been demonstrated [57,58]. In this study, an increase in murine cytokine secretion was associated with a significant decrease in H460 cell viability. Apparently, cytokines detected in our study have interspecies activity, as they affected the human non-small cell lung carcinoma *in vitro*. This interspecies activity of cytokines supports the use of cancer-bearing mouse models to investigate the efficacy of new approaches, such as inhalable NPs, in treating lung cancer.

5. Conclusion

In the present study, DOX-loaded NPs were taken up by alveolar macrophages, and fragments of NPs were released from macrophages over time. The phagocytosis of NPs caused an inflammatory response in macrophages hours after exposure. Normally, an inflammatory reaction caused by a dosage form is an undesirable effect. However, Th1 cytokines, which are secreted by alveolar macro-

phages, caused a significant secondary cytotoxicity effect on H460 cancer cells. Th1 cytokines may add an immune pathway to the chemotherapy approach of DOX-loaded NPs, which may contribute to the overall effectiveness of the treatment *in vivo*.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejpb.2010.05.002.

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