TITLE PAGE

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Quantification of microsized fluorescent particles phagocytosis to a better knowledge of toxicity mechanisms.

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ABSTRACT AND KEY TERMS

Background: The use of micro- or nanometric particles is in full expansion for the development of new technologies. These particles may exhibit variable toxicity levels depending on their physicochemical characteristics. We focused our attention on macrophages, the main target cells of the respiratory system responsible for the phagocytosis of the particles. The quantification of the amount of phagocytosed particles seems to be a major element for a better knowledge of toxicity mechanisms.

The aim of this study was to develop a quantitative evaluation of uptake using both flow cytometry and confocal microscopy to distinguish entirely engulfed fluorescent microsized particles from those just adherent to the cell membrane and to compare these data to *in vitro* toxicity assessments.

Methods: Fluorescent particles of variable and well-characterized sizes and surface coatings were incubated with macrophages (RAW 264.7 cell line). Analyses were performed using confocal microscopy and flow cytometry. The biological toxicity of the particles was evaluated (LDH release, TNF-α and ROS production).

Results and conclusion: Confocal imaging allowed visualization of entirely engulfed beads. The amount of phagocytic cells was greater for carboxylate 2 μm beads (49$\pm$11%) than for amine 1 μm beads (18$\pm$5%). Similarly, Side Scatter (SSC) geometric means, reflecting cellular complexity, were 446$\pm$7 and 139$\pm$12 respectively. These results confirm that the phagocytosis level highly depends on the size and surface chemical groups of the particles. Only TNF-α and global ROS production varied significantly after 24h of incubation. There was no effect on LDH and H_2O_2 production.

Key terms: Phagocytosis quantification, macrophages, fluorescent microsized particles, flow cytometry, confocal microscopy, *in vitro* toxicity evaluations.

INTRODUCTION

The toxicological assessment of fine and ultrafine particles (and particularly nanoparticles) represent a considerable current issue for environmental science, biosciences or nanomedicine (Oberdorster *et al.* 2005; Sayes *et al.* 2007; Warheit *et al.* 2004). In order to better understand the toxicity of particles it seems of high interest to determine the number of internalized particles into the cells after exposure (Clift *et al.* 2008; Haberzettl *et al.* 2007). Different particle entry pathways exist in the human body: skin, gastro-intestinal tract, respiratory tract or by injection. We particularly focus our attention on the respiratory way and its main target cells: the macrophages (MA). These cells are major performers of phagocytosis, a highly conserved complex process, classically defined as the internalization and destruction of particles greater than 0.5 μm in size. It is a receptor-mediated and actin-driven process. Phagocytic uptake involves actin dynamics including polymerization, bundling, contraction, severing and depolymerization of actin filaments (Aderem and Underhill 1999; Fenteany and Glogauer 2004; Niedergang and Chavrier 2004, 2005; Ravetch and Aderem 2007). The physicochemical characteristics of particles may also influence the phagocytic process (Serda *et al.* 2009) and they have also been shown to be involved in the mechanisms of toxicity (Clift *et al.* 2008; Fubini 1998; Fubini *et al.* 2004).

It appears of great interest to quantify the phagocytic process, and compare these data to the toxicity of particles regarding their physicochemical characteristics. Preliminary results have shown that it is impossible to accurately quantify phagocytosis using two dimension (2D) images acquired from cell smears where internalized particles can hardly be distinguished from particles placed at the outer surface of the plasma membrane.

In this context, our work aimed at developing a simple, direct, cost effective, reproducible and easy to perform phagocytosis quantification technique on an *in vitro* MA cell line (RAW

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264.7). The MA were incubated with microsized model particles which are fluorescent beads whose physicochemical characteristics (*i.e.* size and chemical coating) and observations are more easily controlled (Champion *et al.* 2008; Hasegawa *et al.* 2008; Jones *et al.* 2002).

Preliminary confocal observations allowed to distinguish the localization of particles without an accurate quantification of the phagocytic process.

To perform a phagocytosis quantification, we adapted an existing analysis using a trypan blue (TB) quenching in flow cytometry (FCM) (Hed *et al.* 1987; Nuutila and Lilius 2005; Thiele *et al.* 2001; Van Amersfoort and Van Strijp 1994). In addition to its principal function as an exclusion dye of dead cells, TB is known for its ability to "turn off" the green fluorescence emitted by the beads outside the cells. This process allowed us to distinguish internalized beads from those just adhering to the plasma membrane. This approach has been improved by combining Side Scatter measurement with fluorescent mean intensity of beads without and with TB.

Due to the role played by actin filaments for the initiation phase of the phagocytic process, we used different actin polymerization inhibitors such as Cytochalasin-D (Cyt-D) (Haberzettl *et al.* 2007; Kanno *et al.* 2007; Moller *et al.* 2002), Latrunculine-A (Lat) (de Oliveira and Mantovani 1988; DeFife *et al.* 1999; Oliveira *et al.* 1996, 1997), Jasplakinolide (Jasp) (Odaka *et al.* 2000) to block cytoskeleton rearrangements and thus phagocytosis. Cells were also fixed with paraformaldehyde (PFA) to prevent phagocytosis.

Moreover, the toxicity of particles was evaluated using different parameters: Tumor Necrosis Factor alpha (TNF-α) production (to assess the inflammatory response), Lactate Dehydrogenase (LDH) release (reflecting integrity of the cell membrane) and the production of total Reactive Oxygen Species (ROS), especially H_2O_2 (Bruch *et al.* 2004; Catelas *et al.* 1999; Donaldson *et al.* 2002; Sayes *et al.* 2007). This part aimed at assessing if the two types of beads (1 μm amine or 2 μm carboxylate) led to different levels of toxicity.

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Following this methodology we looked for a relationship between toxicity response and the level of phagocytosis determined by confocal microscopy and FCM analysis.

MATERIAL AND METHODS

In vitro model

Macrophage cell culture

The RAW 264.7 cell line was provided by ATCC Cell Biology Collection (Promochem LGC) and derives from mice peritoneal macrophages (MA) transformed by the AMLV virus (Abelson Murine Leukemia Virus). Cells were cultured in DMEM medium (Dulbecco's Modified Eagle's Medium, Gibco) complemented with 10% of fetal calf serum (FCS, Gibco), 1% penicillin-streptomycin (penicillin 10 000 units/mL, streptomycin 10 mg/mL, Sigma) and incubated at 37°C under a 5% carbon dioxide humidified atmosphere.

Fluorescent polymer particles (beads)

- P beads: fluorescent polystyrene particles of 2 μm in diameter with carboxylate coating (Fluoresbrite®YGcarboxylate microspheres, Polyscience). These particles have an excitation peak at 441 nm, and an emission peak at 486 nm (green fluorescence) with a large spectrum.

- I beads: fluorescent polystyrene particles of 1 μm in diameter with amine coating (YGFluoSpheres®amine-modified microspheres, Invitrogen). These particles have an excitation peak at 505 nm, and an emission peak at 515 nm (green fluorescence).

Bead exposure conditions

For the two types of beads, an arbitrary ratio of 10 beads for one cell (10/1¢) has been defined on the basis of the MA size comparatively to bead sizes.

Moreover in order to work with similar surface area for the two types of beads, we used the ratio 40 I beads/1¢ (surface of P beads being four times more important than surface of I beads).

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Actin inhibitors and blocking of phagocytosis

Three actin inhibitors were used: Cyt-D (Sigma), Lat (VWR), Jasp (VWR). They were directly added to cell cultures and incubated at 37°C for 60 minutes, before addition of beads. These molecules remained in the culture medium throughout all the experiments. The concentrations used (5 µM Cyt-D, 0.1 µM Lat and 1 µM Jasp) were defined depending on literature data or preliminary assays.

To block completely the phagocytic process cells were fixed in a 4% PFA solution (Sigma) for 10 minutes before adding P or I beads (Moon *et al.* 2007).

Confocal microscopy phagocytosis assay

Exposure conditions

Cells were grown in 6 well plates (1 million cells in 4 mL) containing coverslips and beads were incubated for 16 hours.

Actin cytoskeleton fluorescent labeling

After the particle exposure, the coverslips were fixed in wells with 4% PFA for 10 minutes (except for the cells already fixed). The wells were washed with Phosphate Buffered Saline (PBS, VWR) to remove beads that did not adhere to the cell membrane or were not internalized. Then actin cytoskeleton was labeled with AlexaFluor®594 phalloidin (Invitrogen; diluted 1:40 in PBS) for 20 minutes and rinsed with PBS. Thereafter cell nuclei were stained with 10 µM of Hoechst 33342 solution (Sigma), during 15 minutes and rinsed with PBS.

Analysis

Microscopic analyses were performed on a confocal microscope TCS-SP2 AOBS (Leica®). Images were obtained from the fluorescence emitted by the beads (ex: 488 nm; em: 499-540 nm), AlexaFluor®594 phalloidin (ex: 543 nm; em: 615-693 nm) and Hoechst (ex: 350 nm; em: 408-443 nm). Confocal microscopy allowed to make stacks of images. Indeed, for a given area of analysis, it was possible to acquire images in different planes of the Z axis (depth of field), to obtain a series of images to achieve XY projections and XZ and YZ section stacks of the selected area of interest.

Quantification of phagocytosis by flow cytometry

FCM exposure conditions and trypan blue quenching

Cells were prepared in 25 cm^2 culture flasks (2 millions cells in 4 mL). A control flask without particles was used to assess the autofluorescence of cells. Some flasks were directly exposed to beads, others were pre-treated with actin inhibitors or PFA before adding P or I beads.

After 90 minutes or 24h of exposure, culture medium was removed, cells were harvested with a scraper and aliquots containing 500 000 cells were centrifuged (10 minutes, 1500 rpm). Cells were resuspended either in 500 µL of DMEM or in 250 µL of DMEM + 250 µL of TB (0.4%, Sigma).

Cytotoxicity was also assessed by adding 1 mg/mL of propidium iodide (Sigma) in the tubes without TB (Jacobs and Pipho 1983; Riccardi and Nicoletti 2006; Steinkamp *et al.* 2000) This evaluation allows to quantify the percentage of dead cells. An exclusion window was made on propidium iodide negative cells to analyze only living cells and quantify the dynamic process of phagocytosis.

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Cytometric analysis

Each condition was analyzed twice on 10 000 cells. Acquisitions and analyses were performed using a cytometer analyzer-cell-sorter FACS Diva™ (BD Biosciences, CA) equipped with the software Cell Quest™ Pro.

The fluorochromes used were excited through an Enterprise II Argon Laser (wavelength set at 488 nm). The fluorescence emitted by P or I beads after excitation at 488 nm was collected through a band pass filter 530/30 nm (FL1 channel) and the fluorescence emitted by excitation of propidium iodide at 488nm was collected through a band pass filter 695/40 nm (FL3 channel). For each acquisition, the size level of detection corresponding to the Forward Scatter (FSC) was set above the size of cellular debris to detect only well preserved cells.

Three FCM parameters were quantified for each sample:

- the average Side Scatter (SSC), morphological parameter reflecting granularity and intracellular complexity, in relation to the presence of both adherent and phagocytosed beads, and quantified by the geometric mean (Y Geo Mean).

- the total Mean Fluorescence Intensity (MFI) corresponding to the total fluorescence emitted by the P or I beads (internalized and adhered) that we called total MFI. These values were obtained for the condition without TB on FL1 channel.

- the percentage of cells with internalized P or I beads that we called "percentage of phagocytic cells" detected on the FL3 channel after TB quenching.

Toxicity assessment

For each experiment, cells were prepared in 96-well plates (100 000 cells/well for TNF-α and LDH assays, and 300 000 cells/well for ROS and H_2O_2 parameters) in 25 µL of complete DMEM. Suspensions of beads in a volume of 75 µL of DMEM were added to the culture and incubated for 90 min or 24h at 37°C in a 5% CO_2 atmosphere.

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TNF-α production

After incubation with beads, released TNF-α was assessed in the supernatant using a commercial ELISA Kit (Quantikine® Mouse TNF-α Immunoassay, R&D Systems) according to the manufacturer's instructions. The optical density of each well was determined using a microplate reader (Multiskan RC, Thermolabsystems) set to 450 nm. A standard curve was established and results were expressed in pg/mL of TNF-α.

LDH release

The activity of the lactate dehydrogenase (LDH) released from cells with damaged membranes was assessed using the CytoTox-ONE™ Homogeneous Membrane Integrity Assay (Promega) according to the manufacturer's instructions. Detection was performed on a fluorometer (Fluoroskan Ascent, Thermolabsystems), using excitation/emission wavelengths at 530/590 nm. The activity of the released LDH was reported to that of total cellular LDH (measured after control cells lysis) and was expressed as a percent of the control.

Oxydative stress

Total ROS: a large array of ROS activity can be assessed with the OxiSelect™ ROS Assay Kit (Cell Biolabs). The assay employs the conversion of a non fluorescent substrate, 2', 7'-Dichlorodihydrofluorescin diacetate (DCFH-DA), which can easily diffuse through cell membranes, into a fluorogenic molecule DCF (2', 7'-Dichlorodihydrofluorescein) which is highly fluorescent and proportional to total ROS level. DCF production was detected using a Fluoroskan Ascent fluorometer (Thermolabsystems) using excitation and emission wavelengths of 480 nm and 530 nm respectively and expressed as nM per hour.

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Specific Hydrogen Peroxide (H_2O_2): after incubation, the cells were stimulated to release the hydrogen peroxide produced by addition of 100 ng/ml phorbol-12-myristate-13-acetate (PMA, Sigma). The release of H_2O_2 was measured as described by De la Harpe (De la Harpe and Nathan 1985). Briefly, KRPG buffer containing a mixture of scopoletin (30 µM), NaN3 (1 mM) and horseradish peroxidase (1 unit pupurogallin/ml HPO) was added to the cells. The horseradish peroxidase catalyzed the oxidation of the fluorescent scopoletin which was measured over a period of 90 min (Fluoroskan Ascent, Thermolabsystems) using excitation/emission wavelengths at 355/460 nm. Results are given as arbitrary units of the fluorescence.

Statistical analysis

Analysis and graphics were performed on Prism 5.0 software (GraphPad, San Diego, CA). Significance was established with ANOVA test ($p < 0.05$).

RESULTS

Confocal microscopy phagocytosis assay

We first verified that the beads were internalized using confocal microscopy imaging (Fig. 1). Figure 1A and 1B confirm that the phalloidin labeling was highly efficient to label actin cytoskeleton (red), beads were easily viewable (green) and Hoechst specifically labeled the nuclei (blue).

Thanks to the XZ and YZ sections of confocal stacks illustrated in Figure 1 (C-a) and (D-a), we demonstrated that beads were internalized beyond question. Indeed beads were surrounded by the phalloidin labeling of actin. On the contrary in Figure 1 (C-b) and (D-b), beads observed at the intersection of the white lines were just on the external side of the cell membrane showing the first step of internalization. Therefore, confocal microscopy allowed us to discriminate beads position (inside/outside).

The same types of results were seen with I beads (data not shown).

FCM phagocytosis quantification assay

The first parameter evaluated in FCM was the Side Scatter (SSC) reflecting morphological cellular complexity (Y Geo Mean). Figure 2 illustrates SSC cytograms (representative of three independent experiments) obtained for cells incubated alone or in presence of P or I beads. The SSC parameter dramatically increased for cells in contact with P beads (446±7 vs. 140±18 for cells alone) (Fig. 2A), whereas it didn't vary significantly for cells in contact with I beads (139±12 vs. 140±18). To verify that beads were internalized by phagocytosis, we used different inhibitors of this process (Cyt-D, Lat, Jasp) and studied the impact on the SSC parameter as shown by Fig. 2B. For cells in contact with P beads, Cyt-D and Lat led to a discrete decrease of the SSC parameter (446±7 vs. 245±2 and 243±2). In presence of Jasp or PFA the SSC parameter was significantly reduced (446±7 vs. 64±2 and

40±2). The same types of results were seen for cells in contact with I beads, Jasp and PFA induced a decrease of the SSC parameter (respectively 139±12 vs. 56±2 and 38±3) though with Cyt-D and Lat there was no effect on the SSC parameter (139±12 vs. 143±1 and 127±3).

In an attempt to quantify the phagocytic process we adapted to our model an existing analysis using trypan blue (TB) quenching to differentiate internalized beads from those just adhering to the cell membrane surface. First, cells were incubated without TB and the total Mean Fluorescence Intensity (MFI) reporting the fluorescence emitted by both ingested and membrane-associated (not-yet-ingested) beads was measured. Free beads have been eliminated by washing steps and were not counted as part of the MFI values. MFI measured for I beads was significantly lower than that measured for P beads (respectively 390±44 and 782±63) for the same ratio of beads (10/1¢). MFI decreased significantly for P beads in presence of Cyt-D (782±63 vs. 564±56) but was still equivalent for I beads (352±21). The MFI decreased sharply for both types of beads in presence of PFA (782±63 vs. 75±5 and 390±44 vs. 96±5). For the analysis with TB, the percentage of phagocytic cells was more important after incubation with P beads than with I beads (49±11% and 18±5% of phagocytic cells respectively). Treatment with Cyt-D resulted in a decrease in the percentage of phagocytic cells (16±3% vs. 49±11% for P beads and 10±1% vs. 18±5% for I beads). According to these data ranges, figure 3 illustrates the results of one among the four independent experiments. Figure 3A shows results without TB (total MFI) and figure 3B presents values after TB quenching.

The effect of these inhibitors and that of Lat and Jasp are summarized in Figure 4. For P beads, Cyt-D and Jasp induced a decrease of the percentage of phagocytic cells close to 50%, whereas Lat seemed to have no impact. For I beads, all inhibitors seemed to have no effects. Finally, whatever the beads employed, a PFA fixation significantly reduced the percentage of phagocytic cells < 2±0.6% ($p < 0.0001$) (Figure 3B).

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Moreover, we verified that the inhibitors (Cyt-D, Lat and Jasp) were not toxic at the concentrations used: the amount of dead cells determined by propidium iodide incorporation was found between 8 and 15% (data not shown). Therefore these inhibitors did not appear to be cytotoxic under these experimental conditions.

Toxicity assessment

The four toxicological parameters evaluated in this study are presented on Figure 5.

Fig 5A clearly shows that the only significant variation in TNF-α concentration was observed when P beads were incubated for 24h with MA. There was no effect when cells were incubated with I beads, neither for 90 min nor for 24h, even for the 40 I beads for one cell ratio. These data could be correlated with the quantification of phagocytosis summarized in Table 1. P beads were more phagocytosed after 24h of incubation (74±5 vs. 49±11) and globally more phagocytosed than I beads (18±5 or 45±6). I beads at a 40/1¢ ratio for 24h were as phagocytosed as P beads at a 10/1¢ ratio after a 24h contact.

No effect was observed concerning the percentage of released LDH showed on Fig 5B. All values were at the same level than the negative control cells alone compared to the positive control (lysed cells) which was significantly different.

The DCF production (nM/h) reflecting the amount of global ROS generated (Fig 5C) indicates a significant enhancement after a 24h incubation for the two types of beads. Correlated with Table 1, these data indicate that ROS generation is linked to the amount of phagocytosis, and incubation time (24h).

Finally H_2O_2 production did not show significant differences among the different conditions of exposure (Fig 5D). After a 24h exposure ROS are generated probably due to H_2O_2 which are likely the source of other organo-peroxydes or lead to formation of hydroxides (Fig 5C).

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DISCUSSION

This study aimed at observing and quantifying phagocytosis using confocal microscopy coupled to FCM. Using these approaches we were able to distinguish entirely engulfed fluorescent micro-sized particles from those just adhering to the cell membrane and FCM appeared as a powerful tool to perform a multiparametric analysis of this cellular process.

Confocal microscopic experiments were very useful to visualize bead internalization. This complementary approach confirms the efficiency of the actin labeling with phalloidin which leads to a precise localization of beads throughout cells. A limit to this technique is that we could not quantify beads on images. Image analyzing techniques could be adapted to this type of acquisition but the area analyzed would be limited. Another technique allowing to quantify the physical process of internalization is necessary. This is the reason why we developed FCM in this study.

 The multiparametric FCM approach we developed was shown to be a powerful tool for the quantification of phagocytosis. This technique allows to analyze an important number of cells without requiring the physical observation of events. Different steps have been necessary to validate this approach especially the use of different phagocytosis inhibitors. FCM also confirmed the effectiveness of the "TB quenching" for quantifying phagocytosis. The results obtained after TB quenching are in good agreement with the dynamics of the phagocytic process and validated the use of TB quenching for quantification in FCM. We have jointly observed that green fluorescence shut down by TB was detected on the FL3 channel (red fluorescence). Indeed, the TB was excited at 488 nm and emitted in the red spectrum. This red fluorescence quenching could be allowed by a process similar to FRET (fluorescence resonance energy transfer) (Haas 2005; He *et al.* 2004).

SSC parameter reflects cellular complexity inside cells but also at the membrane surface, it appears to be a good indicator of cellular dynamics during the process of bead

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internalization. SSC values increased with the amount of internalized beads (P beads). Moreover, we observed SSC changes after treating cells with actin inhibitors used as controls.

This FCM technique allowed us to observe significant differences between the two types of beads which size and coating were different (P beads had a diameter of 2 μm and carboxylate surface groups while I beads had a diameter of 1 μm and amine surface groups). We observed that with P beads the SSC parameter was strongly increased compared to control cells, we quantified 60% of phagocytic cells and the effect of actin inhibitors was important. This suggests that P beads are highly phagocytosed, much more than a same ratio of I beads (10/1¢) for which SSC values were equivalent to that of the control cells, only 20% of phagocytic cells were observed and for which the inhibitors had no effect.

We have shown that Cyt-D, Jasp and Lat did not completely block the internalization of the beads (inhibition of the phagocytosis of P beads up to 50% and no effect on I beads), suggesting that other internalization pathways may be involved and particularly a non actin-driven process (Aderem and Underhill 1999; Castellano *et al.* 2001; Etienne-Manneville and Hall 2002; Gratton *et al.* 2008; Haberzettl *et al.* 2007; Lanzetti 2007; Niedergang and Chavrier 2004, 2005; Yeung and Grinstein 2007). It would be interesting to explore other pathways such as energy-dependent phagocytosis, clathrin-mediated endocytosis, caveolae-mediated endocytosis or macropinocytosis (Gratton *et al.* 2008). Finally, the study of membrane receptors such as the Scavenger Receptors (SR) MARCO (Macrophage Receptor with collagenous structure) and the complement receptor 3 (CR3) implicated in these pathways could also bring further information (Arredouani *et al.* 2004, 2005; Groves *et al.* 2008; Hamilton *et al.* 2006; Kanno *et al.* 2007; May *et al.* 2000; Palecanda and Kobzik 2001; Sulahian *et al.* 2008).

Taken together, these results underline the importance of the physicochemical properties of the particles for the phagocytic process (size and surface groups) (Ahsan *et al.* 2002).

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Further investigations are needed to determine if these two parameters have the same influence or if one prevails and which one (using beads of different size with the same surface groups or conversely beads of same size but carrying different surface groups like carboxylate, amine and polyethylene glycol).

Similarly, these characteristics could have a major impact on the biological toxicity. Toxicological evaluations show that in a global manner, ROS and LDH productions do not depend on the size and chemical composition of beads. Generation of global ROS seems more dependant of time incubation and amount of internalized beads. On the contrary, these parameters play a significant role in TNF-α inflammatory response: carboxylate 2 μm beads (P beads) dramatically increase TNF-α production (Wilson *et al.* 2007), whereas amine 1 μm beads (I beads) seem to have no impact (10/1 and 40/1 ratio). These results fit adequately since we demonstrated that P beads were more phagocytosed than I beads (10/1 ratio) but as much phagocytosed as I beads for a 40/1 ratio reflecting equivalence in surface area. Thus relationship between surface chemistry and TNF-α production is clear.

Conclusion

We established a multiparametric method of analysis of phagocytosis coupling flow cytometry to confocal microscopy. This approach allowed to highlight that physicochemical characteristics of beads (size, surface area and surface coating) were key factors of the internalization process and that they also have an impact on toxicity.

DECLARATION OF INTEREST

The authors report no declarations of interest.

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FIGURE CAPTIONS

Figure 1: Confocal microscopy images of MA incubated with beads (10/1¢ ratio) for 16 hours. A-B) XY projection stack mean of the interest area with phalloidin and Hoechst labeled MA in contact with P beads. C-D) XZ and YZ section stacks with observed beads at the intersection of the white lines (a: bead inside, b: bead outside).

Figure 2: SSC parameter analyzed for MA alone or after a 90 minutes incubation with P or I beads (10/1¢ ratio) (A), and in presence of inhibitors of phagocytosis (Cyt-D, Lat and Jasp) or after PFA fixation (B). The value indicated on each cytogram corresponds to the Y Geo Mean which represents cellular complexity (representative of three independent experiments).

Figure 3: FCM analysis of cells without (A) or with trypan blue (B) after a 90 minutes incubation with P and I beads (10/1¢ ratio) in presence or absence of Cyt-D and PFA. A) Total MFI is represented on the FL1 channel (green fluorescence of all beads). B) FL1 channel detects the green fluorescence of beads internalized by MA. After TB quenching non internalized beads emit a red fluorescence that can be detected on the FL3 channel allowing to distinguish phagocytic (FL1+/FL3-) from non phagocytic (FL3+) cells (illustration representative of four independent experiments).

Figure 4: Effects of inhibitors of phagocytosis (Cyt-D, Lat and Jasp) and PFA fixation on the MA in contact with P or I beads (10/1¢ ratio) analyzed in FCM after 90 min of exposure. The percentage of phagocytic cells is normalized with the control (n=4, *p<0.0001).

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Figure 5: Toxicity assessment for the different conditions : 10/1¢ ratio for P and I beads (after 90 min or 24h contact with RAW 264.7 cells) and 40/1¢ ratio for I beads (after 24h contact) in terms of inflammation assessed by TNF-α production (pg/ml) (A), cell damage measured by the amount of LDH released (B), total ROS (C) and H_2O_2 specific production (D) (n=3, *p<0.0001).

REFERENCES

Aderem, A., and Underhill, D. M. (1999). Mechanisms of phagocytosis in macrophages. Annu Rev Immunol 17, 593-623.

Ahsan, F., Rivas, I. P., Khan, M. A., and Torres Suarez, A. I. (2002). Targeting to macrophages: role of physicochemical properties of particulate carriers-liposomes and microspheres--on the phagocytosis by macrophages. J Control Release 79, 29-40.

Arredouani, M., Yang, Z., Ning, Y., Qin, G., Soininen, R., Tryggvason, K., and Kobzik, L. (2004). The scavenger receptor MARCO is required for lung defense against pneumococcal pneumonia and inhaled particles. J Exp Med 200, 267-72.

Arredouani, M. S., Palecanda, A., Koziel, H., Huang, Y. C., Imrich, A., Sulahian, T. H., Ning, Y. Y., Yang, Z., Pikkarainen, T., Sankala, M., Vargas, S. O., Takeya, M., Tryggvason, K., and Kobzik, L. (2005). MARCO is the major binding receptor for unopsonized particles and bacteria on human alveolar macrophages. J Immunol 175, 6058-64.

Bruch, J., Rehn, S., Rehn, B., Borm, P. J., and Fubini, B. (2004). Variation of biological responses to different respirable quartz flours determined by a vector model. Int J Hyg Environ Health 207, 203-16.

Castellano, F., Chavrier, P., and Caron, E. (2001). Actin dynamics during phagocytosis. Semin Immunol 13, 347-55.

Catelas, I., Petit, A., Marchand, R., Zukor, D. J., Yahia, L., and Huk, O. L. (1999). Cytotoxicity and macrophage cytokine release induced by ceramic and polyethylene particles in vitro. J Bone Joint Surg Br 81, 516-21.

Champion, J. A., Walker, A., and Mitragotri, S. (2008). Role of Particle Size in Phagocytosis of Polymeric Microspheres. Pharm Res 25(8) 1815-21.

Clift, M. J., Rothen-Rutishauser, B., Brown, D. M., Duffin, R., Donaldson, K., Proudfoot, L., Guy, K., and Stone, V. (2008). The impact of different nanoparticle surface chemistry and size on uptake and toxicity in a murine macrophage cell line. Toxicol Appl Pharmacol 232, 418-27.

De la Harpe, J., and Nathan, C. F. (1985). A semi-automated micro-assay for H2O2 release by human blood monocytes and mouse peritoneal macrophages. J Immunol Methods 78, 323-36.

De Oliveira, C. A., and Mantovani, B. (1988). Latrunculin A is a potent inhibitor of phagocytosis by macrophages. Life Sci 43, 1825-30.

DeFife, K. M., Jenney, C. R., Colton, E., and Anderson, J. M. (1999). Disruption of filamentous actin inhibits human macrophage fusion. Faseb J 13, 823-32.

Donaldson, K., Brown, D., Clouter, A., Duffin, R., MacNee, W., Renwick, L., Tran, L., and Stone, V. (2002). The pulmonary toxicology of ultrafine particles. J Aerosol Med 15, 213-20.

Etienne-Manneville, S., and Hall, A. (2002). Rho GTPases in cell biology. Nature 420, 629-35.

Fenteany, G., and Glogauer, M. (2004). Cytoskeletal remodeling in leukocyte function. Curr Opin Hematol 11, 15-24.

Fubini, B. (1998). Surface chemistry and quartz hazard. Ann Occup Hyg 42, 521-30.

Fubini, B., Fenoglio, I., Ceschino, R., Ghiazza, M., Martra, G., Tomatis, M., Borm, P., Schins, R., and Bruch, J. (2004). Relationship between the state of the surface of four commercial quartz flours and their biological activity in vitro and in vivo. Int J Hyg Environ Health 207, 89-104.

Gratton, S. E., Ropp, P. A., Pohlhaus, P. D., Luft, J. C., Madden, V. J., Napier, M. E., and DeSimone, J. M. (2008). The effect of particle design on cellular internalization pathways. Proc Natl Acad Sci USA 105, 11613-8.

Groves, E., Dart, A. E., Covarelli, V., and Caron, E. (2008). Molecular mechanisms of phagocytic uptake in mammalian cells. Cell Mol Life Sci 65, 1957-76.

Haas, E. (2005). The study of protein folding and dynamics by determination of intramolecular distance distributions and their fluctuations using ensemble and single-molecule FRET measurements. Chemphyschem 6, 858-70.

Haberzettl, P., Duffin, R., Kramer, U., Hohr, D., Schins, R. P., Borm, P. J., and Albrecht, C. (2007). Actin plays a crucial role in the phagocytosis and biological response to respirable quartz particles in macrophages. Arch Toxicol 81, 459-70.

Hamilton, R. F., Jr., Thakur, S. A., Mayfair, J. K., and Holian, A. (2006). MARCO mediates silica uptake and toxicity in alveolar macrophages from C57BL/6 mice. J Biol Chem 281, 34218-26.

Hasegawa, T., Iijima, K., Hirota, K., Nakajima, T., Makino, K., and Terada, H. (2008). Exact determination of phagocytic activity of alveolar macrophages toward polymer microspheres by elimination of those attached to the macrophage membrane. Colloids Surf B Biointerfaces 63, 209-16.

He, L., Wu, X., Meylan, F., Olson, D. P., Simone, J., Hewgill, D., Siegel, R., and Lipsky, P. E. (2004). Monitoring caspase activity in living cells using fluorescent proteins and flow cytometry. Am J Pathol 164, 1901-13.

Hed, J., Hallden, G., Johansson, S. G., and Larsson, P. (1987). The use of fluorescence quenching in flow cytofluorometry to measure the attachment and ingestion phases in phagocytosis in peripheral blood without prior cell separation. J Immunol Methods 101, 119-25.

Jacobs, D. B., and Pipho, C. (1983). Use of propidium iodide staining and flow cytometry to measure anti-mediated cytotoxicity: resolution of complement-sensitive and resistant target cells. J Immunol Methods 62, 101-8.

Jones, B. G., Dickinson, P. A., Gumbleton, M., and Kellaway, I. W. (2002). The inhibition of phagocytosis of respirable microspheres by alveolar and peritoneal macrophages. Int J Pharm 236, 65-79.

Kanno, S., Furuyama, A., and Hirano, S. (2007). A murine scavenger receptor MARCO recognizes polystyrene nanoparticles. Toxicol Sci 97, 398-406.

Lanzetti, L. (2007). Actin in membrane trafficking. Curr Opin Cell Biol 19, 453-8.

May, R. C., Caron, E., Hall, A., and Machesky, L. M. (2000). Involvement of the Arp2/3 complex in phagocytosis mediated by FcgammaR or CR3. Nat Cell Biol 2, 246-8.

Moller, W., Hofer, T., Ziesenis, A., Karg, E., and Heyder, J. (2002). Ultrafine particles cause cytoskeletal dysfunctions in macrophages. Toxicol Appl Pharmacol 182, 197-207.

Moon, I. S., Cho, S. J., Jin, I., and Walikonis, R. (2007). A simple method for combined fluorescence in situ hybridization and immunocytochemistry. Mol Cells 24, 76-82.

Niedergang, F., and Chavrier, P. (2004). Signaling and membrane dynamics during phagocytosis: many roads lead to the phagos(R)ome. Curr Opin Cell Biol 16, 422-8.

Niedergang, F., and Chavrier, P. (2005). Regulation of phagocytosis by Rho GTPases. Curr Top Microbiol Immunol 291, 43-60.

Nuutila, J., and Lilius, E. M. (2005). Flow cytometric quantitative determination of ingestion by phagocytes needs the distinguishing of overlapping populations of binding and ingesting cells. Cytometry A 65, 93-102.

Oberdorster, G., Oberdorster, E., and Oberdorster, J. (2005). Nanotoxicology: an emerging discipline evolving from studies of ultrafine particles. Environ Health Perspect 113, 823-39.

Odaka, C., Sanders, M. L., and Crews, P. (2000). Jasplakinolide induces apoptosis in various transformed cell lines by a caspase-3-like protease-dependent pathway. Clin Diagn Lab Immunol 7, 947-52.

Oliveira, C. A., Chedraoui, S., and Mantovani, B. (1997). Latrunculin A is a potent inducer of aggregation of polymorphonuclear leukocytes. Life Sci 61, 603-9.

Oliveira, C. A., Kashman, Y., and Mantovani, B. (1996). Effects of latrunculin A on immunological phagocytosis and macrophage spreading-associated changes in the F-actin/G-actin content of the cells. Chem Biol Interact 100, 141-53.

Palecanda, A., and Kobzik, L. (2001). Receptors for unopsonized particles: the role of alveolar macrophage scavenger receptors. Curr Mol Med 1, 589-95.

Ravetch, J., and Aderem, A. (2007). Phagocytic cells. Immunol Rev 219, 5-7.

Riccardi, C., and Nicoletti, I. (2006). Analysis of apoptosis by propidium iodide staining and flow cytometry. Nat Protoc 1, 1458-61.

Sayes, C. M., Reed, K. L., and Warheit, D. B. (2007). Assessing toxicity of fine and nanoparticles: comparing in vitro measurements to in vivo pulmonary toxicity profiles. Toxicol Sci 97, 163-80.

Serda, R. E., Gu, J., Burks, J. K., Ferrari, K., Ferrari, C., and Ferrari, M. (2009). Quantitative mechanics of endothelial phagocytosis of silicon microparticles. Cytometry A 75, 752-60.

Steinkamp, J. A., Valdez, Y. E., and Lehnert, B. E. (2000). Flow cytometric, phase-resolved fluorescence measurement of propidium iodide uptake in macrophages containing phagocytized fluorescent microspheres. Cytometry 39, 45-55.

Sulahian, T. H., Imrich, A., Deloid, G., Winkler, A. R., and Kobzik, L. (2008). Signaling pathways required for macrophage scavenger receptor-mediated phagocytosis: analysis by scanning cytometry. Respir Res 9, 59.

Thiele, L., Rothen-Rutishauser, B., Jilek, S., Wunderli-Allenspach, H., Merkle, H. P., and Walter, E. (2001). Evaluation of particle uptake in human blood monocyte-derived cells in vitro. Does phagocytosis activity of dendritic cells measure up with macrophages? J Control Release 76, 59-71.

Van Amersfoort, E. S., and Van Strijp, J. A. (1994). Evaluation of a flow cytometric fluorescence quenching assay of phagocytosis of sensitized sheep erythrocytes by polymorphonuclear leukocytes. Cytometry 17, 294-301.

Warheit, D. B., Laurence, B. R., Reed, K. L., Roach, D. H., Reynolds, G. A., and Webb, T. R. (2004). Comparative pulmonary toxicity assessment of single-wall carbon nanotubes in rats. Toxicol Sci 77, 117-25.

Wilson, M. R., Foucaud, L., Barlow, P. G., Hutchison, G. R., Sales, J., Simpson, R. J., and Stone, V. (2007). Nanoparticle interactions with zinc and iron: implications for toxicology and inflammation. Toxicol Appl Pharmacol 225, 80-9.

Yeung, T., and Grinstein, S. (2007). Lipid signaling and the modulation of surface charge during phagocytosis. Immunol Rev 219, 17-36.

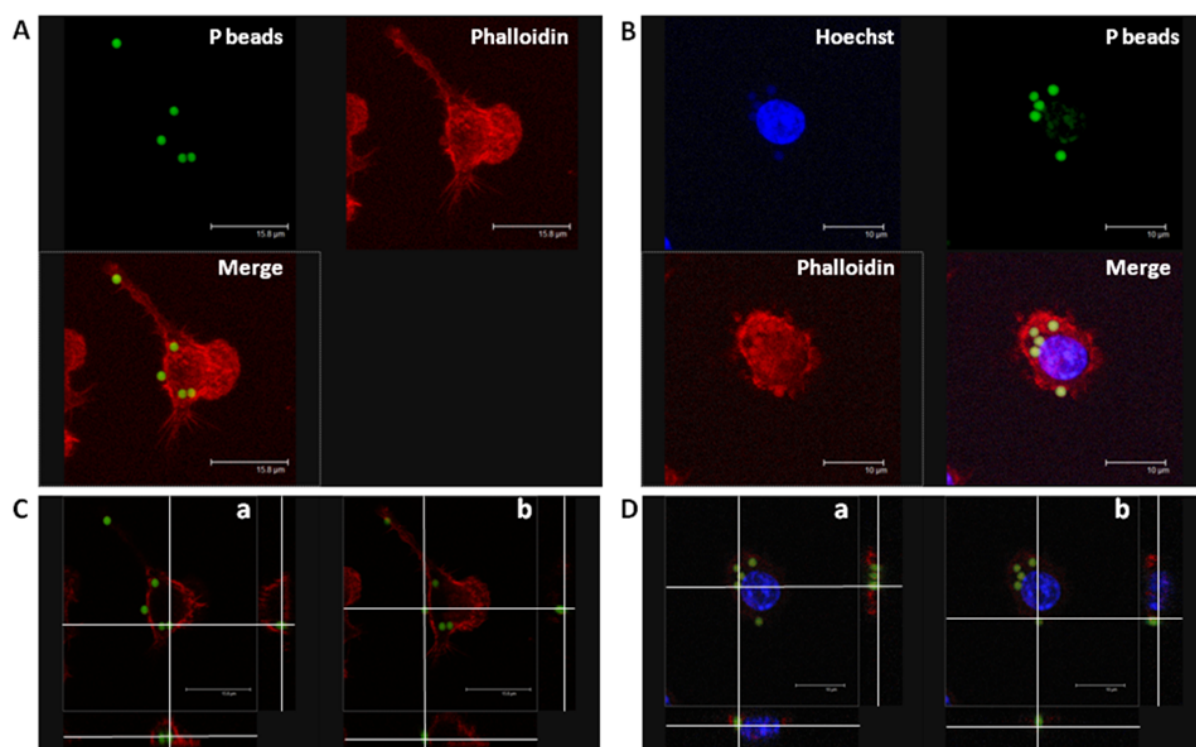Figure 1: Confocal microscopy images of MA incubated with beads (10/1¢ ratio) for 16 hours. A-B) XY projection stack mean of the interest area with phalloidin and Hoechst labeled MA in contact with P beads. C-D) XZ and YZ section stacks with observed beads at the intersection of the white lines (a: bead inside, b: bead outside).
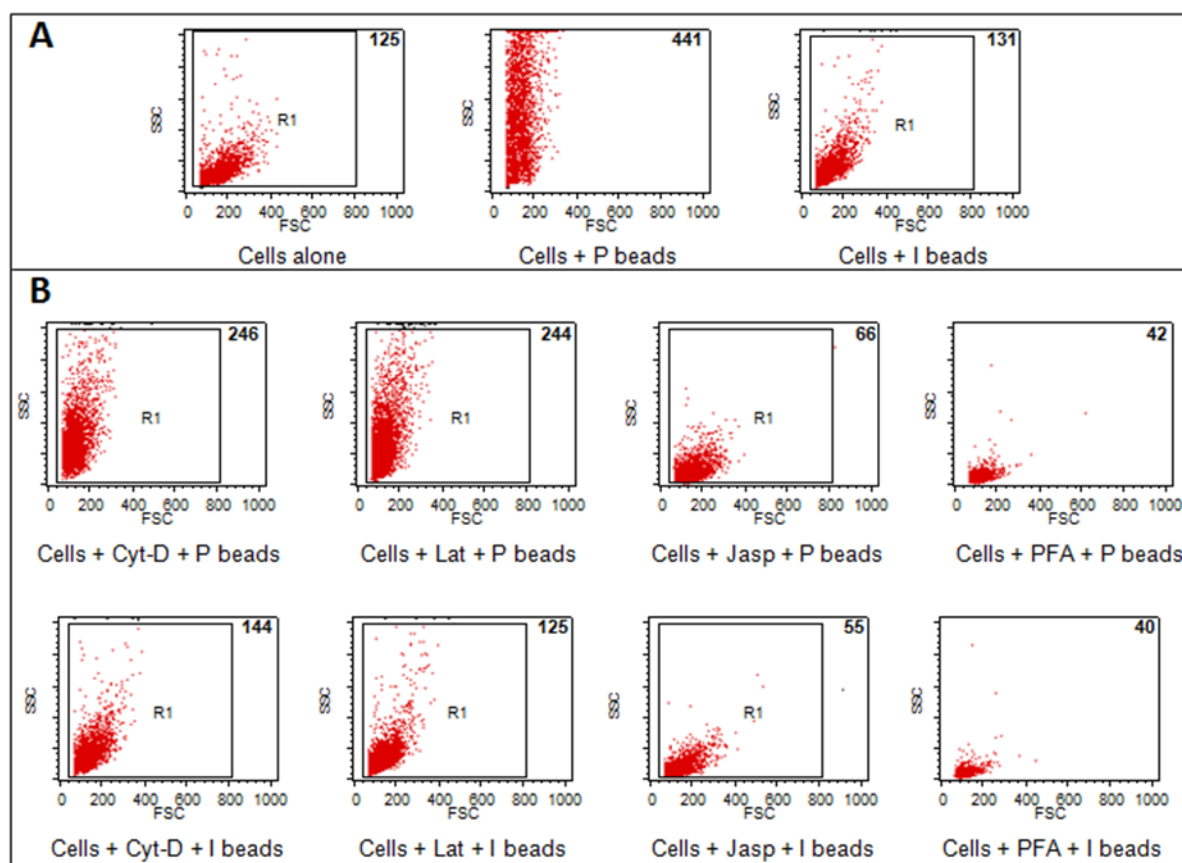
Figure 2: SSC parameter analyzed for MA alone or after a 90 minutes incubation with P or I beads (10/1¢ ratio) (A), and in presence of inhibitors of phagocytosis (Cyt-D, Lat and Jasp) or after PFA fixation (B). The value indicated on each cytogram corresponds to the Y Geo Mean which represents cellular complexity (representative of three independent experiments).
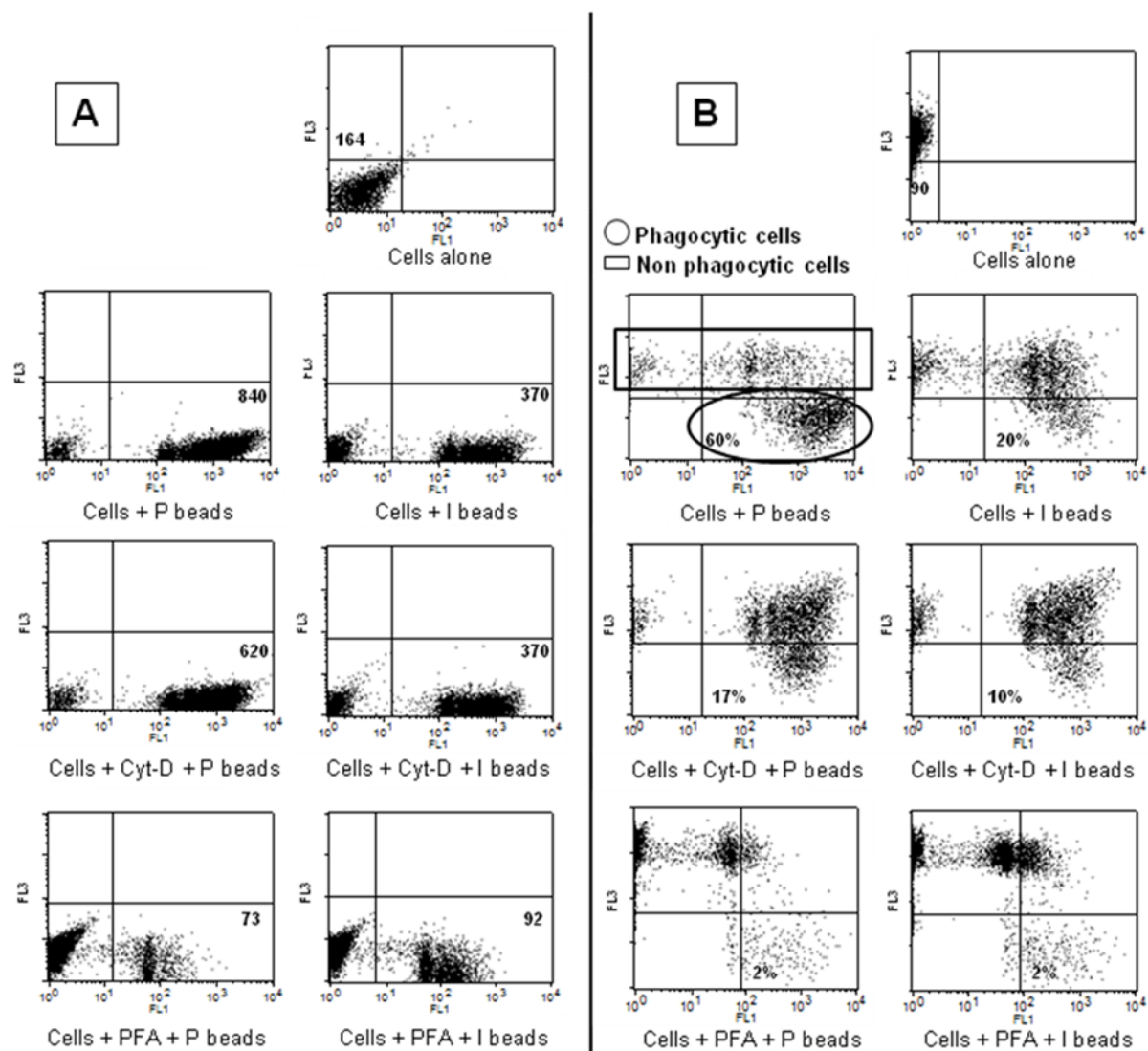
Figure 3: FCM analysis of cells without (A) or with trypan blue (B) after a 90 minutes incubation with P and I beads (10/1¢ ratio) in presence or absence of Cyt-D and PFA. A) Total MFI is represented on the FL1 channel (green fluorescence of all beads). B) FL1 channel detects the green fluorescence of beads internalized by MA. After TB quenching non internalized beads emit a red fluorescence that can be detected on the FL3 channel allowing to distinguish phagocytic (FL1+/FL3-) from non phagocytic (FL3+) cells (illustration representative of four independent experiments).
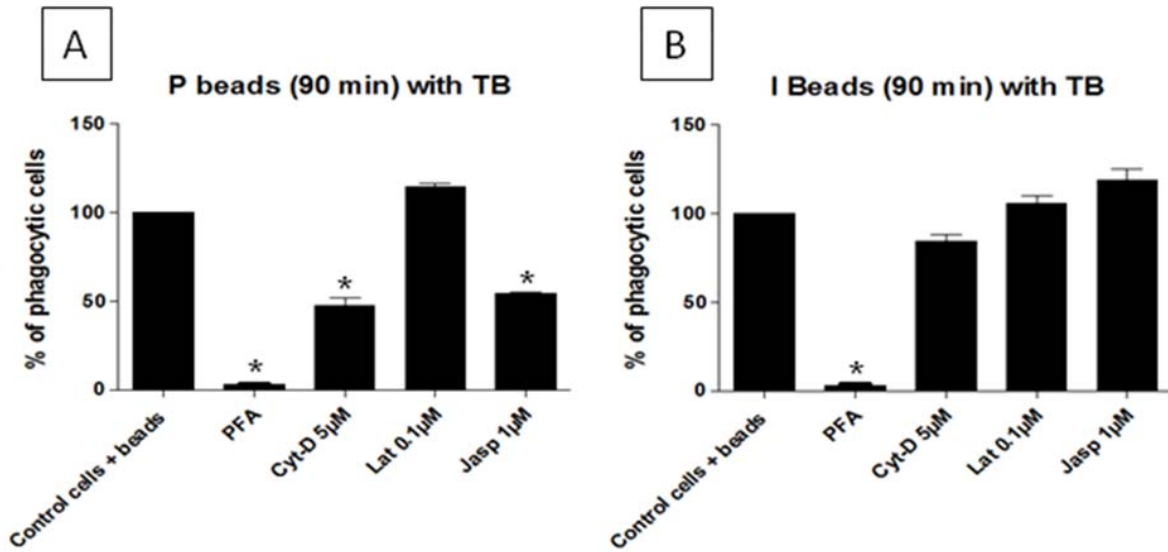
Figure 4: Effects of inhibitors of phagocytosis (Cyt-D, Lat and Jasp) and PFA fixation on the MA in contact with P or I beads (10/1¢ ratio) analyzed in FCM after 90 min of exposure. The percentage of phagocytic cells is normalized with the control (n=4, *p<0.0001).
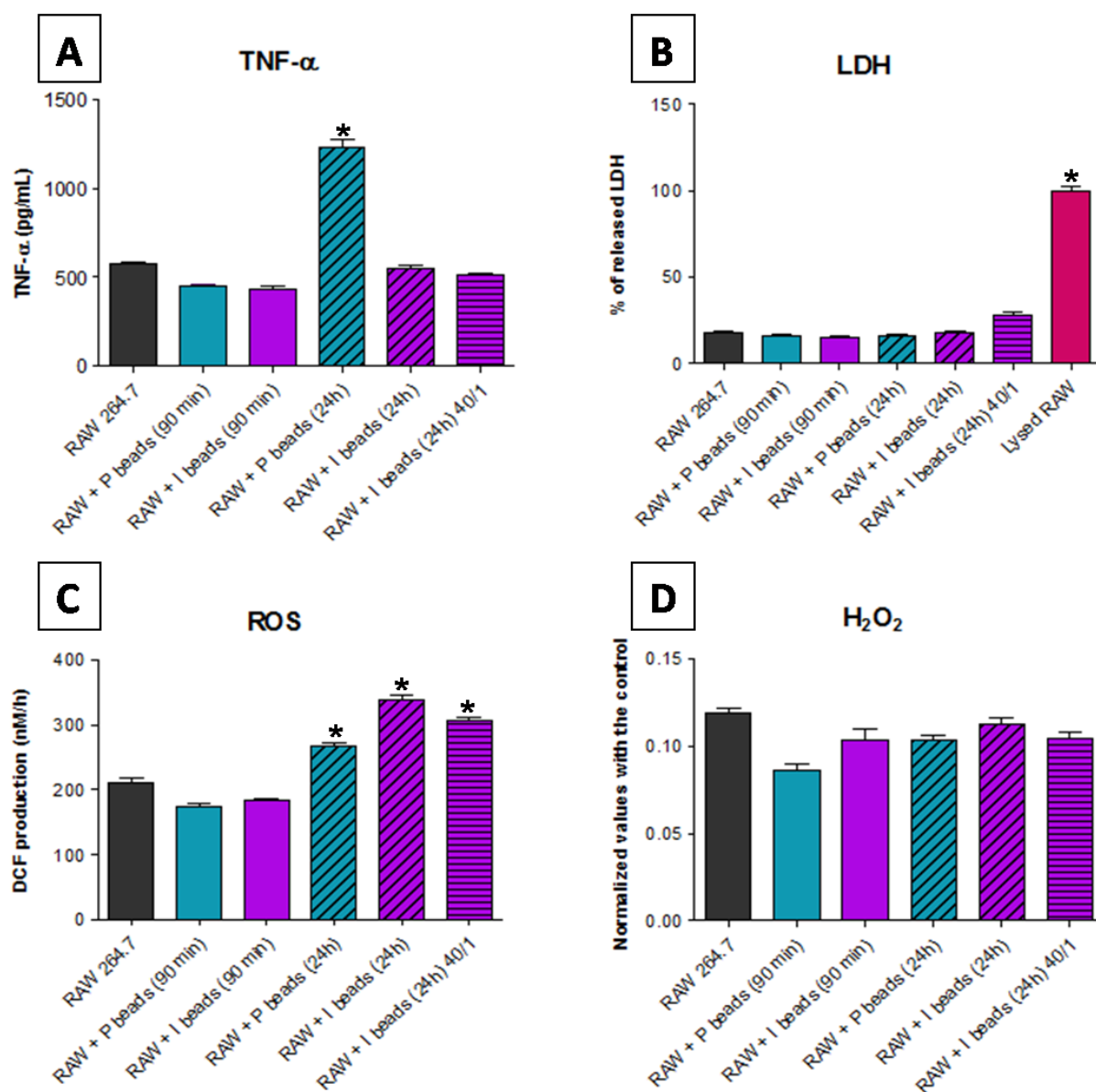
Figure 5: Toxicity assessment for the different conditions : 10/1¢ ratio for P and I beads (after 90 min or 24h contact with RAW 264.7 cells) and 40/1¢ ratio for I beads (after 24h contact) in terms of inflammation assessed by TNF-α production (pg/ml) (A), cell damage measured by the amount of LDH released (B), total ROS (C) and H2O2 specific production (D) (n=3, *p<0.0001).

	P bead incubation		I bead incubation		
	10/1¢		10/1¢		40/1¢
	90 min	24h	90 min	24h	24h
SSC	446±7	649±21	139±12	192±19	183±32
total MFI	782±63	828±75	390±44	508±91	831±48
% of phagocytic cells	49±11	74±5	18±5	45±6	76±6

Table 1: Summary data of quantification parameters obtained in FCM (SSC, total MFI and percentage of phagocytic cells) for the different exposure conditions : 10/1¢ ratio for P and I beads after a 90 min or 24h contacts with RAW 264.7 cells and 40/1¢ ratio for I beads after a 24h contact.