

Human eosinophils are direct targets to nanoparticles: Zinc oxide nanoparticles (ZnO) delay apoptosis and increase the production of the pro-inflammatory cytokines IL-1 β and IL-8



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HIGHLIGHTS

- Nanoparticles can alter the biology of human eosinophils.
- Zinc oxide nanoparticles delay human eosinophil apoptosis.
- Zinc oxide nanoparticles increase the production of pro-inflammatory cytokines.

ARTICLE INFO

Article history:

Received 30 March 2016
Received in revised form 29 June 2016
Accepted 18 July 2016
Available online 21 July 2016

Keywords:

Eosinophils
Nanoparticles
Apoptosis
Caspases
De novo protein synthesis
Cytokines

ABSTRACT

Zinc oxide NPs (ZnO) have been recently proposed as novel candidates for the treatment of allergic inflammatory diseases. Paradoxically, recent data suggested that ZnO could cause eosinophilic airway inflammation in rodents. Despite the above observations, there are currently no studies reporting direct interaction between a given NP and human eosinophils themselves. In this study, freshly isolated human eosinophils were incubated with ZnO and several cellular functions were studied. We found that ZnO delay human eosinophil apoptosis, partially by inhibiting caspases and by preventing caspase-4 and Bcl-xL degradation. ZnO do not induce production of reactive oxygen species but increase de novo protein synthesis. In addition, ZnO were found to increase the production of the proinflammatory IL-1 β and IL-8 cytokines. Using a pharmacological approach, we demonstrated that inhibition of caspase-1 reversed the ability of ZnO to induce IL-1 β and IL-8 production, whereas inhibition of caspase-4 only reversed that of IL-8. Our results indicate the necessity of conducting studies to determine the potential of using NP as nanotherapies, particularly in diseases in which eosinophils may be involved. We conclude that, indeed, human eosinophils represent potential new direct targets to NPs, ZnO in the present case.

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1. Introduction

Eosinophil granulocytes are best known for their role in the host defense against parasitic pathogens and to play a pivotal role in the pathogenesis of asthma and allergic diseases (Shamri et al., 2011). However, increasing evidences revealed additional roles for eosinophils in regulating inflammation, especially by their ability to secrete different mediators, including a variety of cytokines such as IL-1 β and IL-8 (Braun et al., 1993; Shamri et al., 2011). Although eosinophils only account for ~3% of blood leukocytes in humans,

they can accumulate in tissues and airways under certain pathological conditions. Eosinophils are known to undergo spontaneous apoptosis and subsequent elimination by professional phagocytes is thought to be crucial for the resolution of airway inflammation, which occurs in asthma. Several agents are known to prolong eosinophil survival, including the three antiapoptotic cytokines GM-CSF, IL-3 and IL-5 (Ilmarinen and Kankaanranta, 2014; Ilmarinen et al., 2014; Tai et al., 1991). Interestingly, these cytokines are present in the lungs of asthmatic patients (Ilmarinen and Kankaanranta, 2014; Ilmarinen et al., 2014). In contrast, other agents can induce eosinophil apoptosis, including the plant lectin *Viscum album* agglutinin-1 (Lavastre et al., 2005) and glucocorticoids (Druihe et al., 2003), these latter being very effective anti-inflammatory drugs used in the treatment of eosinophilic

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disorders such as asthma (Barnes, 1996). Therefore, identification of new agents that can modulate eosinophil apoptosis represents an important step for the development or improvement of therapeutic strategies that target eosinophils.

A tremendous interest to use nanomaterials for medical diagnosis and therapeutic purposes has increased in the past few years. Although a lot of investigations focus on the use of NPs for drug delivery in cancer therapies, there are several studies promoting the use of NPs as carriers to detect allergies or to alleviate inflammatory symptoms (Szelenyi, 2012). However, even this represents a very interesting interest and a potential avenue to use nanodrug systems, there are some potential toxic risks since, cytotoxicity, oxidative stress, genotoxicity, and inflammation have been reported, both in *in vitro* and *in vivo* models (Onoue et al., 2014). Among nanoparticles, zinc oxide NPs (Zhang et al., 2013) (hereafter denoted as ZnO) are good prospects to be used in nanomedicine. Indeed, ZnO were recently proposed as novel candidates for the treatment of allergic inflammatory diseases (Kim et al., 2014). However, ZnO were found to possess pro-inflammatory activities both *in vitro* and *in vivo* (Babin et al., 2013; Goncalves and Girard, 2014; Roy et al., 2013). For example, based on the fact that exposure to ZnO in environmental and occupational setting causes acute pulmonary responses via induction of pro-inflammatory mediators, including interleukin-8 (IL-8), Wu et al. (2010) demonstrated that ZnO induce IL-8 production by human bronchial epithelial cells via NF- κ B activation. *In vivo*, although topically applied ZnO were found to suppress allergen-induced skin inflammation, these NP also induces vigorous IgE production in the atopic dermatitis mouse model. In addition, it was recently shown that ZnO induce eosinophilic airway inflammation in mice (Huang et al., 2015).

Despite the fact that an increased number of neutrophils and/or eosinophils are observed in different *in vivo* models of inflammation and that most of the *in vitro* studies performed do not use primary human cells, the role of NPs on human granulocyte biology needs to be better documented. To the best of our knowledge, there are no studies reporting a direct interaction with a given NP and human eosinophils. Here, we show that ZnO NPs can alter the biology of human eosinophils by suppressing apoptosis and by inducing the production of the pro-inflammatory cytokines IL-1 β and IL-8.

2. Materials and methods

2.1. Nanoparticles

Zinc oxide nanoparticles used in this study have been described previously (Babin et al., 2013).

2.2. DLS analysis

The size distribution, surface charge (zeta potential) and the polydispersion index (PDI) of the NPs were determined by dynamic light scattering using a Malvern Zetasizer Nano-ZS (model ZEN3600 from Malvern Instruments Inc., Westborough, MA). Measurements were performed at 37 °C with NPs in suspension in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), the medium in which NPs were suspended and incubated with eosinophils throughout this study or in HBSS alone, the medium used for the detection of intracellular ROS as described under.

2.3. Eosinophil isolation

Blood donations were obtained from informed and consenting individuals according to our institutionally approved procedures.

Granulocytes were isolated from venous blood of healthy volunteers by dextran sedimentation followed by centrifugation over Ficoll-Hypaque (GE Healthcare, Uppsala, Sweden), to obtain neutrophils and eosinophils as previously described (Lavastre et al., 2005). Experiments were performed when cell purity was >95% as verified by cytology from cytocentrifuged preparations colored by the Hema 3 Stain Set (Biochemical Sciences Inc., Swedesboro, NJ). Cell viability was verified by trypan blue exclusion and was always >98%. When >5% of eosinophils were detected in granulocyte enriched preparations, eosinophils were separated from neutrophils by negative immunomagnetic selection using anti-human CD16-coated magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's recommendations. Purity and viability after eosinophil isolation were routinely >95% as assessed as above.

2.4. Cell viability and cell shape changes

Freshly isolated human eosinophils (5×10^5 cells/ml in RPMI-1640 HEPES-P/S, supplemented with 10% heat-inactivated FBS) were treated for 0–48 h with or without the indicated concentrations of ZnO. Cell viability was monitored by trypan blue exclusion morphological cell shape changes were observed under light microscopy (100X), and photomicrographs were taken using a Nikon Eclipse TS100 camera.

2.5. Evaluation of cell size and inner complexity by flow cytometry

Eosinophils (5×10^5 cells/ml) were treated with or without 100 μ g/ml ZnO for 30 min, 3 h, 24 h and 48 h and data for cell size (FSC-H) and inner complexity (SSC-H) were acquired by flow cytometry using a FACScan (BD Biosciences) as previously published (Poirier et al., 2014).

2.6. Assessment of eosinophil apoptosis

Apoptosis was evaluated by cytology, as previously published (Lavastre et al., 2005; Poirier et al., 2014). Eosinophils (5×10^5 cells/ml) were incubated at 37 °C in 5% CO₂ in 48-well plates for 24 h in the presence of buffer (Ctrl), the pro-apoptotic plant lectin VAA-I (0.5 μ g/ml) or the indicated concentrations of ZnO. Cells were cytocentrifuged on microscope slides and colored with the Hema 3 staining kit. Apoptosis evaluation was based on the nucleus morphology. The results were expressed as the percentage of eosinophils in apoptosis. In some experiments, annexin-V binding was used to determine the eosinophil apoptotic rate after 24 h. Cells were suspended at concentrations of 5×10^6 cells/mL, washed and then resuspended at 1×10^6 cells/mL and incubated with 2 μ L of FITC-annexin-V for 305 min at 4 °C (light protected) prior to fluorescence-activated cell sorting (FACS) analysis. In some experiments the pancaspase inhibitor (Z-VAD-FMK), the specific caspase-1 (Z-YVAD-FMK) or caspase-4 (Z-LEVD-FMK) inhibitors were added 1 h (all at 50 μ M) prior the addition of ZnO for a total of 24 h before determining the apoptotic rate.

2.7. Western blot analysis

Cells (1×10^6 cells/ml) were incubated with buffer (Ctrl), rhIL-5 (30 ng/ml) or ZnO (50 μ g/ml) for 24 h and then harvested for the preparation of cell lysates in Laemmli's sample buffer. Aliquots corresponding to 500 000 cells were loaded and subjected to 10% SDS-polyacrylamide gel electrophoresis and transferred from the gel to PVDF or nitrocellulose membranes. The membranes were blocked for 1 h at room temperature in 5% milk in TBS-Tween (25 mM Tris-HCl, pH 7.8, 190 mM NaCl, 0.15% Tween-20). After washing, a dilution of 1:1000 was used for BCL-XL antibodies

(Santa Cruz) or caspase-4 (Cell Signaling) in TBS-Tween 0.15%. The membranes were kept overnight at 4 °C, then washed with TBS-Tween, and incubated for 1 h at room temperature with HRP-labeled goat anti-mouse IgG antibodies (Jackson ImmunoResearch, 1:25,000) followed by several washes. Protein expression was revealed using Clarity™ western ECL (Bio-Rad). Membranes were stripped with ReBlot Plus Strong (Millipore) and reprobed with an anti-GAPDH antibody (Santa Cruz, 1:2000) to confirm equal loading of proteins. Chemiluminescence was revealed with a chemiDoc™ MP Imaging system from Bio-Rad.

2.8. Detection of intracellular ROS

Cells (5×10^5 cells/ml) were suspended in HBSS containing 10 μ M CM-H₂DCFDA for 15 min at 37 °C as previously published (Simard et al., 2011). Cells were then washed twice before being incubated in the presence of buffer or 100 μ g/ml ZnO for 5, 15, 30, 60 and 90 min. PMA (10^{-7} M) was used as a positive control. Fluorescence was recorded using a FACScan. ROS production was expressed as MFI.

2.9. Metabolic labeling and de novo protein synthesis assay

Cells (5×10^5 cells/ml) were metabolically labeled with 4.625 MBq of the EXPRESS [³⁵S]-protein labeling mix EasyTag™ (PerkinElmer, Waltham, MA) in the presence or absence of 100 μ g/ml ZnO or the indicated concentrations of cycloheximide (CHX) for 24 h as previously described (Poirier et al., 2014).

2.10. Measurements of IL-1 β and IL-8 by ELISA

The measurement of IL-1 β and IL-8 production in the extracellular milieu was determined using commercially available ELISA kits (Invitrogen Canada Inc., ONT) as per the manufacturer's recommendation. In some experiments the pancaspase inhibitor (Z-VAD-FMK), the specific caspase-1 (Z-YVAD-FMK) or caspase-4 (Z-LEVD-FMK) inhibitors were added 1 h (all at 50 μ M) prior the addition of 100 μ g/ml ZnO for a total of 24 h before quantification of the cytokines.

2.11. Statistical analysis

The data are reported as mean $\pm$ SEM and were analyzed by one-way ANOVA. Differences between tested groups and control

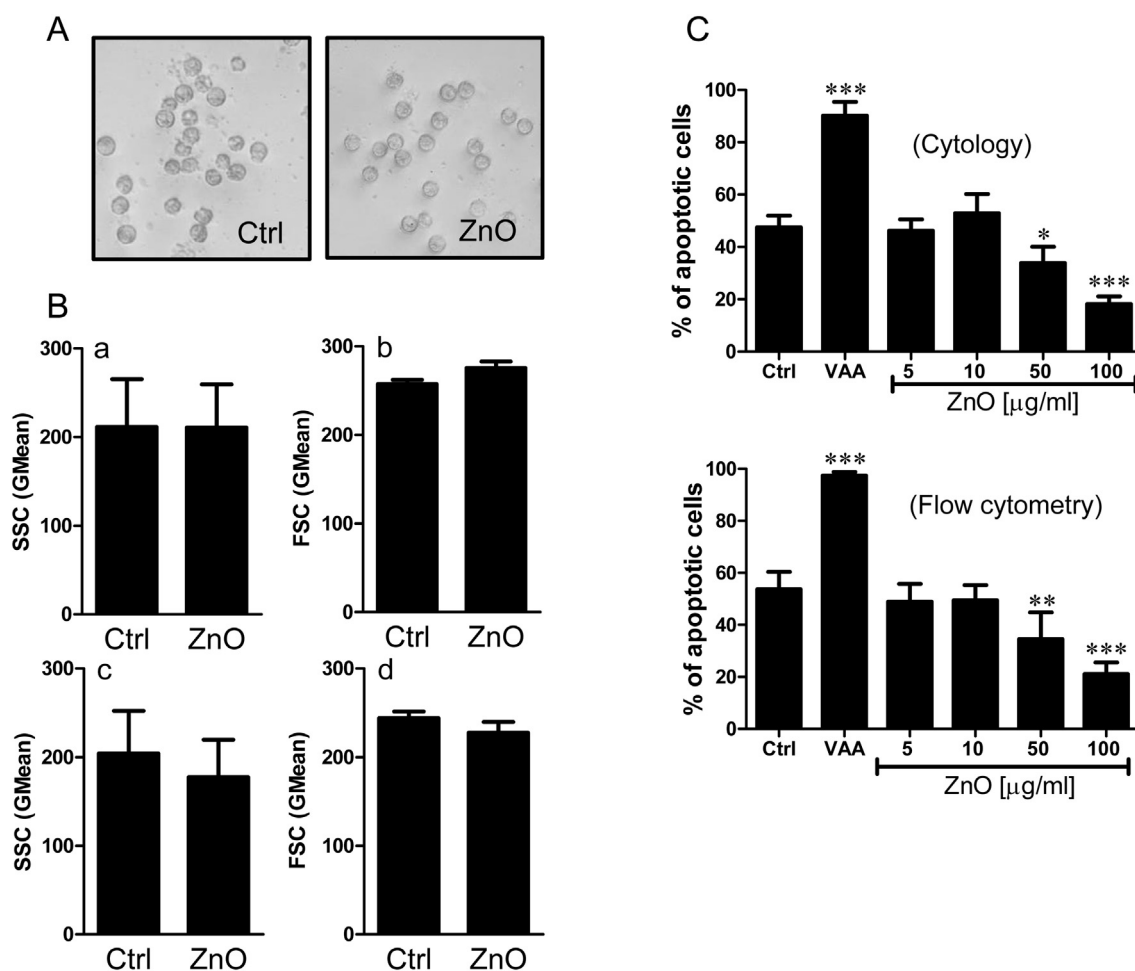


Fig. 1. ZnO do not induce cell shape changes, do not affect cell size and intracellular complexity but delay spontaneous eosinophil apoptosis. (A) freshly isolated human eosinophils were incubated in the presence or absence (Ctrl) of ZnO (100 μ g/ml) for 0–48 h and cell morphology was monitored by optical microscopy. For clarity, only the data obtained after 48 h are shown. Results are typical illustrations representative of 5 different experiments. (B) cells were incubated in the presence or absence (Ctrl) of ZnO (100 μ g/ml) for 30 min (panels a,b) or 3 h (panels c and d) and intracellular granularity (SSC, panels a,c) and cell size (FSC, panels b,d) were determined by flow cytometry. Results are means $\pm$ SEM (n = 3); (C) cells were incubated for 24 h in the presence of buffer (Ctrl), *Viscum album* agglutinin-1 (VAA) or the indicated concentration of ZnO and apoptosis was determined by cytology (upper panel) and flow cytometry (bottom panel) by determining the number of FITC-annexin-V positive cells as described in Materials and Methods. Results are means $\pm$ SEM (n $\geq$ 4).

were assessed using the Dunnett's Multiple Comparison Test using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA). Statistical significance was established at * $p \leq 0.05$; ** $p \leq 0.01$; and *** $p \leq 0.005$ vs control or appropriate diluent, and each experiment was performed and validated at least 3 times using different blood donors.

3. Results

3.1. Characterization of ZnO nanoparticles

As determined by DLS, the size distribution of ZnO in RPMI medium + 10% FBS was trimodal with a size of 61.9 nm (58.8%), 11.6 nm (37.2%) and large aggregates of ~ 3900 nm (4%). The values for the zeta potential and PDI are -8.7 ± 0.8 mV and 0.5 ± 0.04 . However, different values were observed when the NP were suspended in HBSS alone. Indeed, a unimodal size distribution of ZnO of 2.9 nm (100%) was observed. Also, a different zeta potential value of -8.3 ± 0.8 mV was observed with a PDI of 1 ± 0 .

3.2. Eosinophil cell viability, cell shape changes and apoptotic rate in response to ZnO treatment

After 24 h, ZnO did not significantly alter the basal level of cell viability which was $>92\%$ at concentrations ranging from 5 to 100 $\mu\text{g/ml}$ (*data not shown*). However, after 48 h $\sim 50\%$ of

eosinophils were viable in absence of NPs but, in the presence of 50 or 100 $\mu\text{g/ml}$ of ZnO, cell viability increased to at least 70% (*data not shown*). Based on these results, and from those obtained previously when studying the interaction between different NPs and human neutrophils (Babin et al., 2013; Goncalves et al., 2010; Poirier et al., 2014), we used a concentration of 100 $\mu\text{g/ml}$ ZnO throughout this study, otherwise specified. Next, cell shape changes were monitored by cytology from 0 to 48 h after NP treatment. As illustrated in Fig. 1A, the cell shape of ZnO-induced eosinophils was not altered and remained round or spherical. For clarity, only the 48 h time point is shown, since the cell shape did not change throughout the experiments. Because cellular uptake of NPs in mammalian cells can be determined by flow cytometric light scatter analysis (Suzuki et al., 2007) following a treatment within few min to few hours, we next evaluated whether or not human eosinophils were able to internalize ZnO in vitro. As illustrated in Fig. 1B, treatment with ZnO did not alter the granularity in eosinophils (SSC in the graph) when tested after 30 min (panel a), as well as after 3 h (panel c) and even after up to 48 h (*data not shown*), suggesting that ZnO do not enter inside eosinophils. In addition to the granularity, the cell size was determined in parallel by flow cytometry (FSC) and, as illustrated in Fig. 1B (panels c and d), it remained unchanged after 30 min and 3 h, respectively. Just as for the granularity, we did not observe any changes for the cell size over a period of up to 48 h (*data not shown*). Some NPs were recently found to modulate spontaneous apoptosis

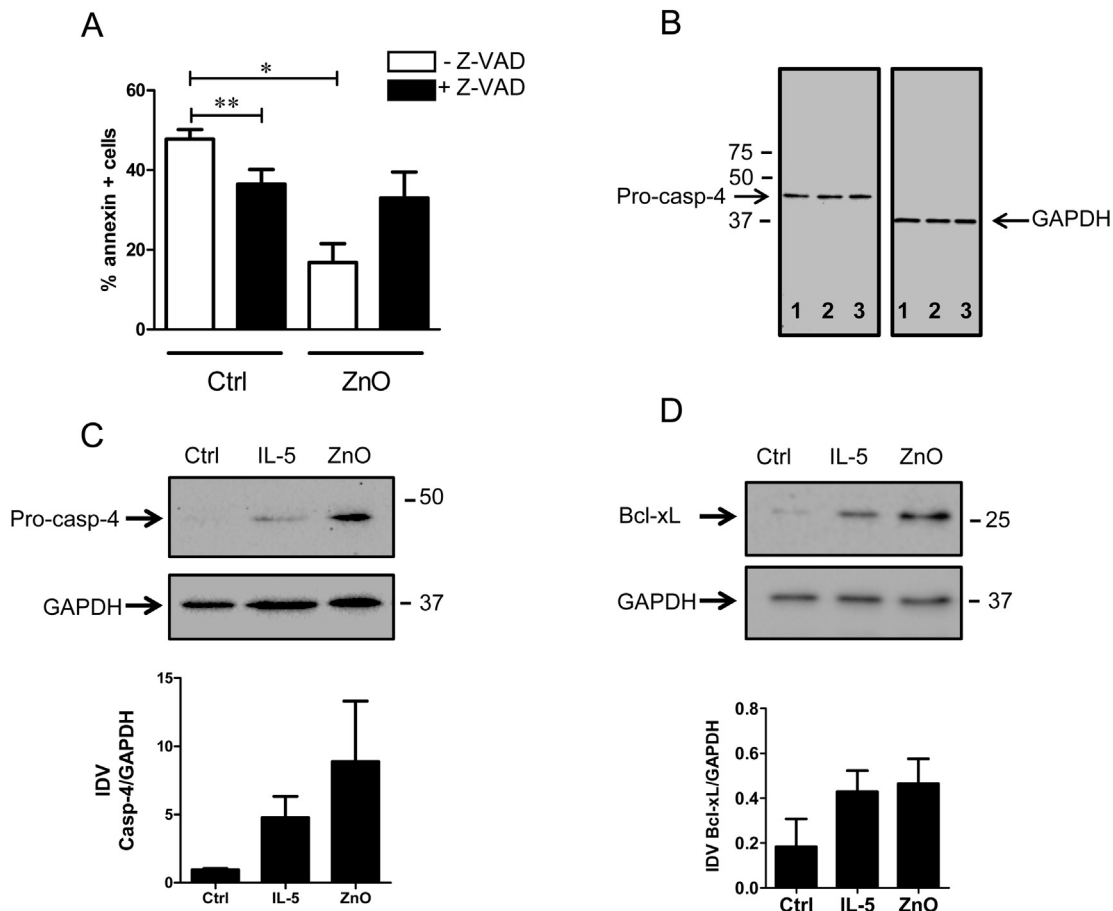


Fig. 2. ZnO inhibit caspase activity in human eosinophils and prevent pro-caspase-4 and Bcl-xL degradation. A, eosinophils were incubated 1 h with (+) or without (–) the pan-caspase inhibitor Z-VAD-FMK (Z-VAD) 1 h prior addition of buffer (Ctrl) or ZnO for a total of 24 h and apoptosis was evaluated by flow cytometry by determining the number of FITC-annexin-V positive cells. B–D, cell lysates were prepared with fresh unstimulated eosinophils (B) or from cells incubated with buffer (Ctrl), IL-5 or ZnO for 24 h and the protein expression of pro-caspase-4 (Pro-casp-4), Bcl-xL or GAPDH (for loading control) were determined by western blot experiments as described in Materials and Methods. A, results are means $\pm$ SEM ($n = 4$). B, lanes 1–3, represent three different blood donors. C and D, (upper part), results are from one representative experiments out of three. Densitometry analysis is illustrated in the corresponding bottom part.

in human neutrophils (Goncalves et al., 2010; Goncalves and Girard, 2014), but their effects on eosinophil apoptosis are still unknown. To verify this, we incubated freshly isolated human eosinophils with increasing concentrations of ZnO and determined the apoptotic rate after 24 h by cytology. The 24 h time point was selected based on the fact that normally, ~30–50% of eosinophils undergo spontaneous apoptosis, a suitable time point for our experimental conditions (Ilmarinen et al., 2009). Fig. 1C (upper panel) illustrates that ZnO inhibited spontaneous eosinophil apoptosis in a concentration-dependent fashion when compared to untreated cells. In these experiments, the plant lectin VAA-1 known to induce human eosinophil apoptosis was used as a positive control (Lavastre et al., 2005). Results were confirmed with another assay measuring the binding of FITC-annexin-V (Fig. 1C, bottom panel).

3.3. ZnO inhibit caspases and prevent Bcl-xL degradation in human eosinophils

In order to determine if ZnO could act by inhibiting caspases, we next used the pancaspase inhibitor Z-VAD-FMK. As illustrated in Fig. 2A, addition of Z-VAD reversed the antiapoptotic effect of ZnO in human eosinophils. In other cells, ZnO were found to be

proapoptotic and to act as an endoplasmic reticulum (ER) stressor, generally involving caspase-4 (Chen et al., 2014). Here, since Z-VAD is known to inhibit caspase-1, -3, -4 and -7, we reasoned that ZnO could inhibit the ER stress-induced cell apoptosis pathway, by preventing caspase-4 activation, which, prior this study, has probably never been investigated in human eosinophils. Although the protein expression of caspase-1 has been recently reported in human eosinophils (Wong et al., 2014), that of caspase-4 is unknown. Therefore, we first determined if these cells express caspase-4 at the protein level. As illustrated in Fig. 2B, human eosinophils express caspase-4. Fig. 2C illustrates that treatment with ZnO prevents the degradation of caspase-4 at different degrees when compared with control cells where caspase-4 was only faintly visible. In these experiments, the antiapoptotic IL-5 cytokine was used in parallel and was also found to prevent caspase-4 degradation. IL-5 is known to delay human eosinophil apoptosis by increasing/maintaining the expression of the potent antiapoptotic protein Bcl-xL (Dibbert et al., 1998). Therefore, we next decided to determine if the mode of action of ZnO will be similar or not. As illustrates in Fig. 2D, the protein expression of Bcl-xL was increased by ZnO when compared to Ctrl, correlating with their antiapoptotic effect in human eosinophils. As expected, IL-5 also increased the Bcl-xL expression.

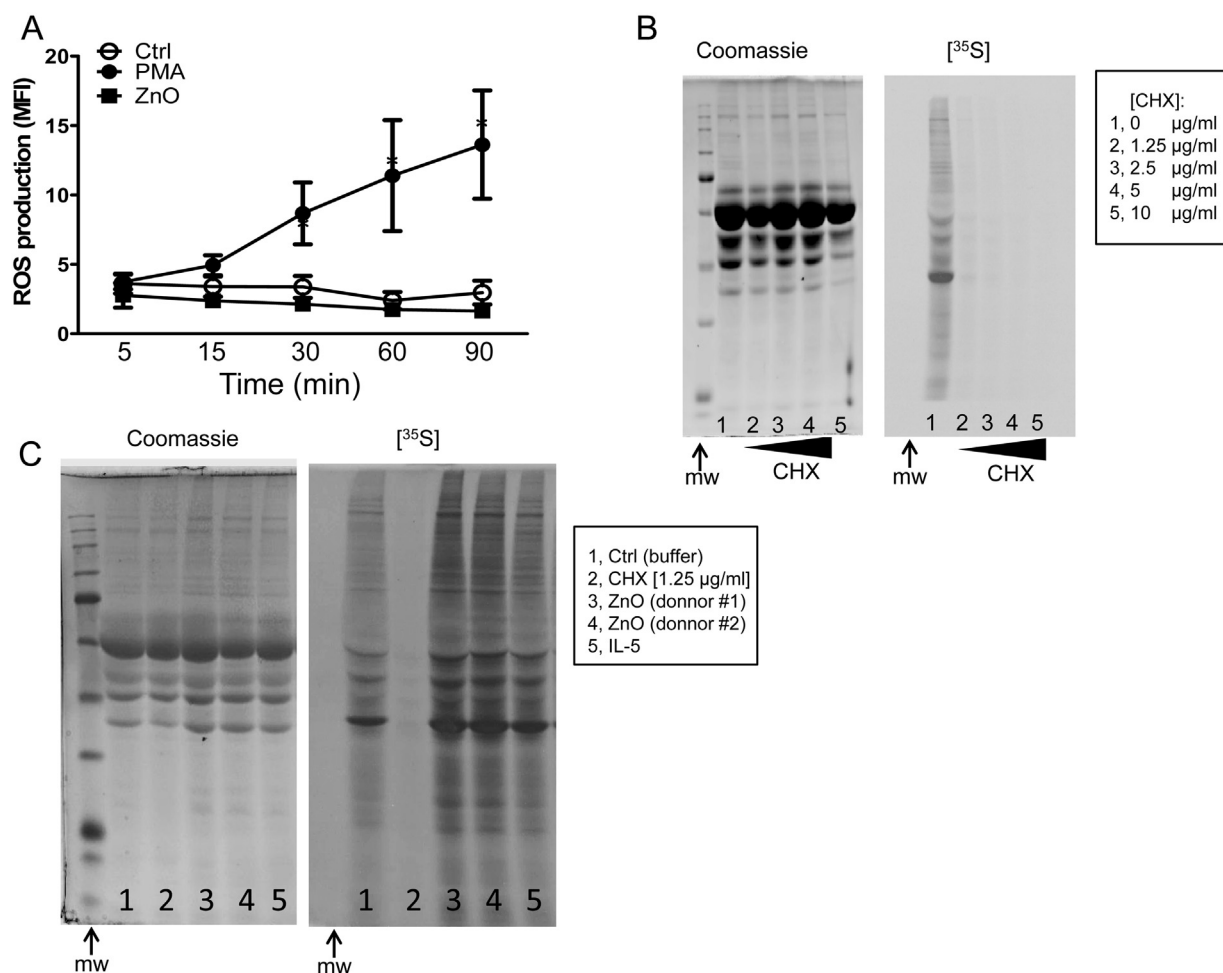


Fig. 3. ZnO do not increase ROS production but induce de novo protein synthesis in human eosinophils. A, cells were stained with the probe CM-H2DCFDA for 15 min and then treated with buffer (Ctrl), PMA, or ZnO for the indicated period of time and ROS production was determined by flow cytometry as described in Materials and Methods. Results are means $\pm$ SEM ($n \geq 4$). B and C, cells were metabolically labeled and incubated with the indicated agents for 24 h and de novo protein synthesis was performed as described in Methods. Results are from one representative experiment out of four. CHX, cycloheximide; mw, molecular weights.

3.4. ZnO NPs do not promote ROS production but induce de novo protein synthesis in human eosinophils

ZnO NPs were previously found to induce ROS production, but this has never been reported in human eosinophils (Song et al., 2010). In this study, as illustrated in Fig. 3A, ZnO did not induce ROS production when compared to untreated cells (0–90 min). Cells were however responsive, since, as expected (Kovacs et al., 2014) PMA induce ROS production. In order to better understand the mode of action of ZnO, we next investigated possible involvement of de novo protein synthesis. We first performed a series of experiments by incubating freshly isolated human eosinophils for 24 h in the presence or absence of an increasing concentration of CHX, a potent protein synthesis inhibitor. Although de novo protein synthesis has been the object of several studies in human neutrophils (Binet et al., 2006; Girard et al., 1997; Goncalves and Girard, 2014; Poirier et al., 2014), this has not been reported in eosinophils. Interestingly, as illustrated in Fig. 3, eosinophils synthesize new polypeptides when incubated without addition of exogenous cytokines and/or growth factors (see Fig. 3B, lane 1). This de novo synthesis of polypeptides is inhibited by CHX in a concentration-dependent fashion (Fig. 3B, lanes 2–5). Next, the effect of ZnO on the ability of human eosinophils to exert de novo protein synthesis was investigated. As illustrated in Fig. 3C, ZnO induce the neo synthesis of several new polypeptides, as strongly as IL-5. Because this is probably the first study investigating the role of de novo protein synthesis during human eosinophil

apoptosis, we first determined how CHX itself will alter spontaneous apoptosis aiming that, as in neutrophils (Binet et al., 2006; Goncalves and Girard, 2014), it will not affect apoptosis. Unfortunately, unlike in neutrophils, CHX significantly induce eosinophil apoptosis in a concentration-dependent manner (Fig. 4A), even at the lowest concentration of 1.25 µg/ml. We normally use a concentration of 10 µg/ml CHX with neutrophils at a cell density of 10×10^6 cells/ml for investigating the role of protein synthesis during apoptosis without altering the spontaneous apoptotic rate. However, we performed here a series of experiments to determine the role of CHX in neutrophils incubated at a lower cell density in order to make comparison with eosinophils. Interestingly, the basal apoptotic rate of human neutrophils incubated at a cell density of 0.5×10^6 cells/ml, was close to 90% and this value decreased to ~70% and ~45% when neutrophils were incubated at 5 and 10×10^6 cells/ml, respectively (Fig. 4B). Addition of 10 µg/ml of CHX during these experiments did not significantly alter neutrophil spontaneous apoptosis.

3.5. The pancaspase Z-VAD-FMK inhibitor reverses the ability of ZnO to induce the production of IL-1β, but not IL-8

IL-8 and IL-β are two important pro-inflammatory cytokines known to be produced by human eosinophils (Braun et al., 1993; Esnault et al., 2012). We next determined whether or not ZnO NPs increase the production of these two cytokines. As illustrated in Fig. 5, ZnO significantly increased the levels of IL-8 (Fig. 5A) and IL-1β (Fig. 5B) detected in the supernatants after 24 h of treatment. We next quantified the IL-1β and IL-8 release in the supernatants of eosinophils treated for 60 min with the pancaspase Z-VAD-FMK inhibitor prior incubation with ZnO. As illustrated in Fig. 5C, the ability of ZnO to increase the production of IL-1β was markedly and significantly inhibited by the pancaspase inhibitor. However, in contrast, the IL-8 production was not significantly affected by the same treatment (Fig. 5D).

3.6. The ZnO-induced production of both IL-1β and IL-8 is reversed by the caspase-1 inhibitor while the caspase-4 inhibitor reverses only the production of IL-1β

Given the importance of caspase-1 in the production of IL-1β (Man and Kanneganti, 2015), and since caspase-4 play important roles in the production of IL-1β by its ability to regulate caspase-1 in humans (Kajiwarra et al., 2014), and that caspase-4 is expressed in human eosinophils (this report), we next verified the possibility that the IL-1β production produced by human eosinophils in response to ZnO could be dependent of caspase-1 and/or caspase-4. Fig. 6 shows that the ability of ZnO to induce the production of IL-1β was inhibited by caspase-1 (panel A) and by caspase-4 (panel B) inhibitors. However, the IL-8 production induced by ZnO was not inhibited by the caspase-1 inhibitor (panel D), whereas it was in the presence of the Z-LEVD-FMK caspase-4 inhibitor (panel E). As expected (Wu et al., 2010), inhibition of NF-κB by Bay 117085 reversed the ZnO-induced IL-8 production (panel C), but also that of IL-1β (panel F).

4. Discussion

In this study, we present the first evidences that NPs, namely ZnO, can alter the biology of human eosinophils. The results of the DLS analysis indicate that the ZnO suspension is homogeneous when suspended in HBSS buffer but not in RPMI ± FCS. However, we cannot conclude if the few (mega)aggregates (4% of the NP suspension) observed when ZnO NP are suspended in RPMI ± FCS are responsible for the biological effects observed. It is important

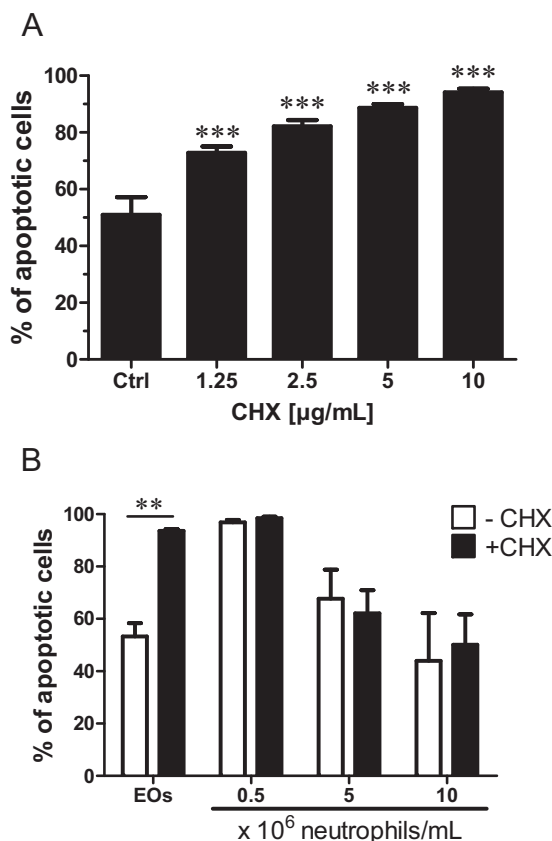


Fig. 4. Cycloheximide induce eosinophil apoptosis and, unlike in neutrophils, could not be used to reverse spontaneous and ZnO-induced suppression of apoptosis. A, eosinophils were incubated in the presence of the indicated concentrations of cycloheximide (CHX) and apoptosis was assessed by cytology after 24 h as described in Materials and Methods. Results are means ± SEM (n = 4). B, eosinophils (EOs) or neutrophils were incubated in the presence or absence of 10 µg/ml CHX and apoptosis was determined by cytology after 24 h at various neutrophil cell density. Results are means ± SEM (n = 4).

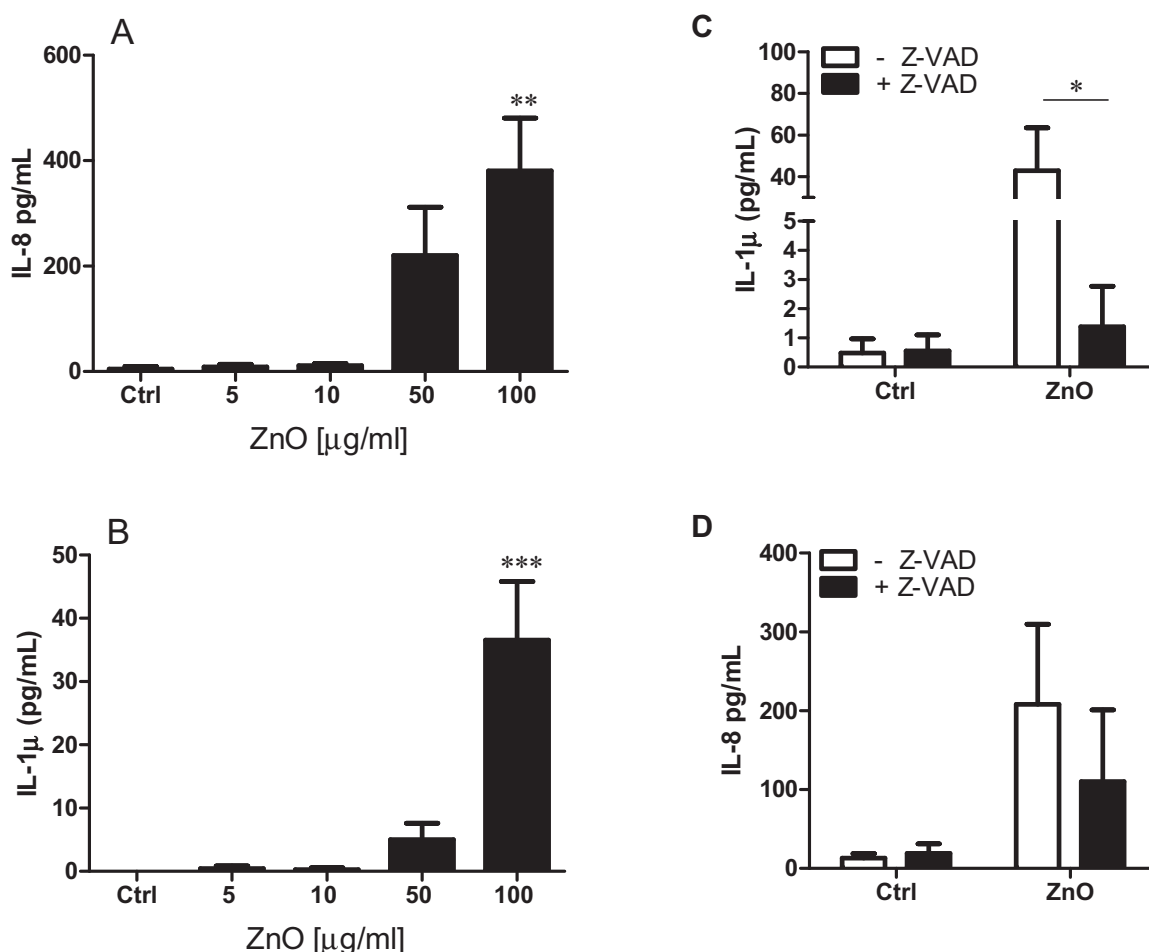


Fig. 5. Role of caspases in the ability of ZnO to induce the production of IL-1 β and IL-8 in human eosinophils. (A, B) eosinophils were incubated in the presence of buffer (Ctrl) or of the indicated concentrations of ZnO for 24 h and the supernatants were collected and used to measure the amount of IL-1 β and IL-8 by ELISA as described in Materials and Methods. (C, D) cells were pre-incubated 1 h with the pan-caspase Z-VAD-FMK (+Z-VAD) inhibitor or its diluent (-Z-VAD) and buffer (Ctrl) or 100 μ g/ml ZnO were added for a total period of time of 24 h and the supernatants were collected and used to measure the amount of IL-1 β and IL-8 by ELISA. Results are means $\pm$ SEM ($n = 5$ for A,B or $n \geq 3$ for C,D).

to remember that 96% of the ZnO NP are under 100 nm and are thus still considered as nanoparticles.

Indeed, we found that ZnO delay eosinophil apoptosis. These results are in contrast with the vast majority of studies investigating the cytotoxic effects of ZnO on cells others than eosinophils. For example, at concentrations of 10 and 50 μ g/ml (identical to us), ZnO were found to have proapoptotic effects on rat subcutaneous adipose-derived mesenchymal stem cells (Orazizadeh et al., 2015), human pulmonary adenocarcinoma cell line LTP-a-2 (Wang et al., 2015), rat astrocyte C6 glial cell line (Sruthi and Mohanan, 2015), human hepatocyte HepG2 cells (Sharma et al., 2012; Wahab et al., 2014) and human breast cancer MCF-7 cells (Wahab et al., 2014). However, in agreement with our present results, ZnO were found to inhibit apoptosis in isolated rat pancreatic islets (Shoae-Hagh et al., 2014) and, as we recently documented, also in primary human neutrophils (Goncalves and Girard, 2014). Although our results regarding the granularity determined by flow cytometry suggested that ZnO were not ingested by human eosinophils, we are aware that further experiments need to be performed to make any firm conclusion. Interestingly, although the major mechanism of ZnO-induced apoptosis in a variety of cells appears to be associated with the production of ROS (Akhtar et al., 2012; De Berardis et al., 2010; Wang et al., 2014), we found here in human eosinophils that ZnO do not induce ROS production. This is in agreement with Shoae-

Hagh et al. (2014) reporting that ZnO even decrease ROS production, using the non-fluorescent compound DCFH-DA (2',7'-dichlorodihydrofluorescein diacetate), a probe similar to CM-H₂DCFDA that we used here as a general oxidative stress indicator. Also in agreement with our present results, we have previously reported that ZnO do not induce ROS production in human neutrophils (Goncalves and Girard, 2014). Since different proapoptotic agents are known to activate caspases, here we reasoned that inhibition of caspases with a general caspase inhibitor (Z-VAF-FMK) will reverse the antiapoptotic effect of ZnO in human eosinophils. In this study, we also demonstrate that these cells express caspase-4 at the protein level and that its inhibition also prevented the antiapoptotic effect of ZnO, opening the possibility that these NP alleviate a pre-existing ongoing ER stress-induced cell apoptotic machinery. In parallel, we also reported here that ZnO prevent the degradation of Bcl-xL, a crucial player known to be involved in the antiapoptotic effect mediated by the cytokine IL-5 (and GM-CSF) (Dibbert et al., 1998). Therefore, our results indicate that the antiapoptotic properties of ZnO are complex as they involve caspase inactivation as well as they prevent Bcl-xL degradation.

To the best of our knowledge, this is the first study showing that human eosinophils can perform de novo protein synthesis, especially in response to NP. Even if our results indicate that ZnO increase the synthesis of neo-polypeptides, CHX is not suitable

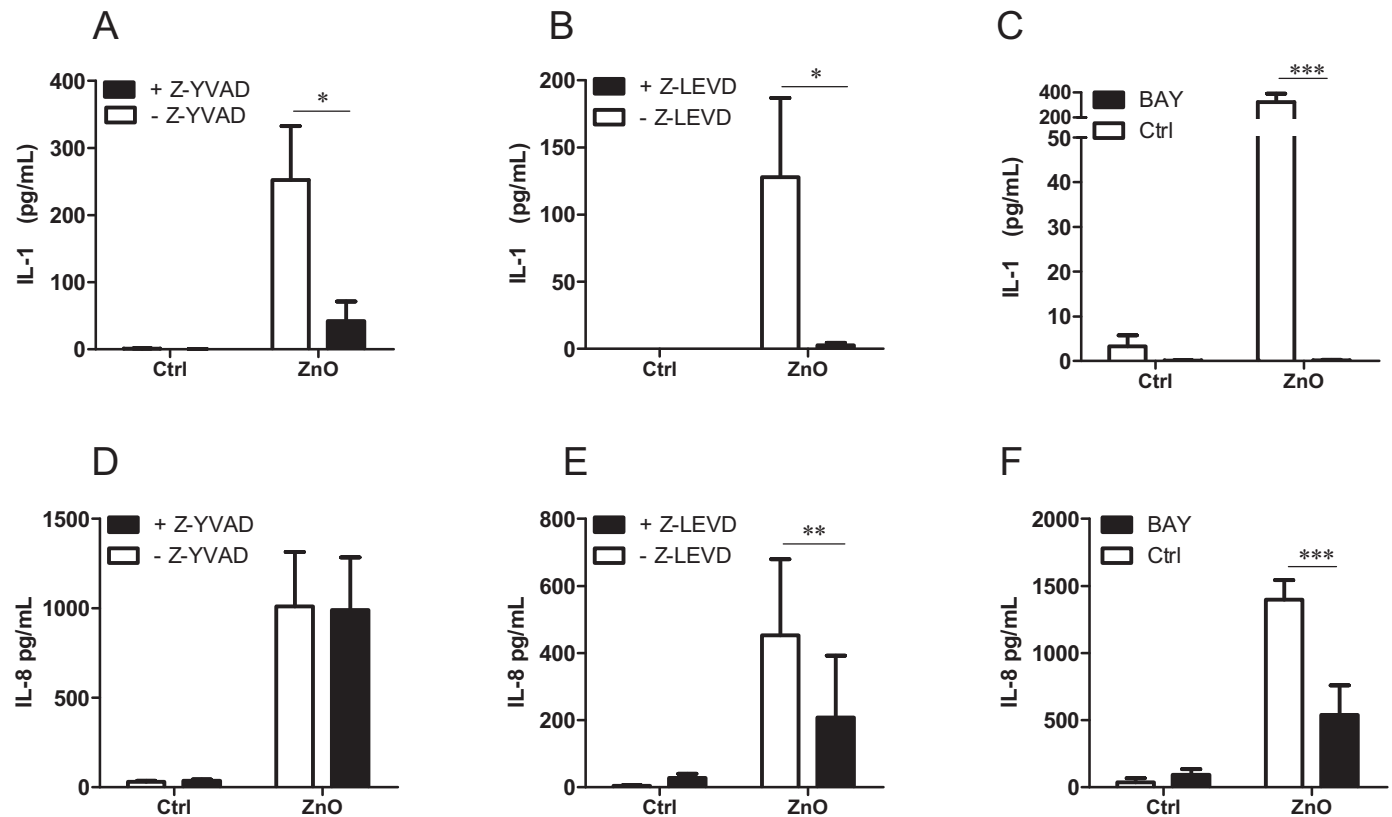


Fig. 6. Effect of caspase-1 and caspase-4 inhibitors on the ZnO-induced production of IL-1 β and IL-8 in human eosinophils. Eosinophils were pre-incubated 1 h with (+) or without (–) the caspase-1 (Z-YVAD), caspase-4 (Z-LEVD) or NF- κ B (BAY) inhibitors and buffer (Ctrl) or 100 μ g/ml ZnO were added for a total period of time of 24 h. The concentrations of IL-1 β and IL-8 were measured by ELISA as described in Materials and Methods. Results are means $\pm$ SEM ($n \geq 3$).

for investigating the role of de novo protein synthesis during apoptosis in human eosinophils, as opposed to human neutrophils. Indeed, CHX by itself was found to induce apoptosis in eosinophils but has no significant effect when used in human neutrophils at the same cell density of 0.5×10^6 cells/ml. It is interesting to note that ZnO delay apoptosis and induce de novo protein synthesis in both cell types, but the profiles of proteins involved in these two situations remained to be determined. Thus, based on these two aspects (delay of apoptosis and induction of de novo protein synthesis) nanoparticles like ZnO can act similarly to other agents, including methyl mercuric chloride (Moisan et al., 2003), sodium butyrate (Stringer et al., 1996), or IL-4 cytokine (Girard et al., 1997). The fact that eosinophils undergoing spontaneous apoptosis (in absence of exogenous agents like ZnO in this study or IL-5) can synthesize new polypeptides fits well with a possible activation of an unfolding protein response following an ER stress, a situation when cells attempt to overcome apoptosis by producing several proteins, especially chaperones. However, other studies are needed to fully understand this and also to determine the mode of action of ZnO in human eosinophil apoptosis.

Herein, ZnO were also found to induce the production of both IL-1 β and IL-8 in human eosinophils, two cytokines important in inflammation. Because of the increasing recognized roles of caspases in the production of cytokines, it was important to determine here if caspases are somewhat involved in ZnO-induced cytokine production. This was determined with the use of the pan caspase-inhibitor Z-VAD-FMK where the production of IL-1 β and IL-8 was decreased drastically and weakly, respectively. Caspase-1 is among the caspases known to be inhibited by Z-VAD-FMK, and this caspase is of particular interest for its role during

inflammasome activation and IL-1 β production (Man and Kanne-ganti, 2015). Caspase-4 is also inhibited by Z-VAD-FMK and is also somewhat involved in inflammasome activation and IL-1 β production (Vigano et al., 2015). Recently, caspase-4 was found to regulate caspase-1 activity in humans (Kajiwaru et al., 2014) but, to the best of our knowledge, its role in human eosinophils has not been documented and its presence at the protein level had not been reported before the present study. Although there are some data regarding inflammasome activation in a murine model of ovalbumin-induced allergic airway inflammation where active caspase-1, and some inflammasome components, were detected from mice tissue eosinophils (Tran et al., 2012), activation of inflammasome leading to the IL-1 β production in human eosinophils is unknown. Therefore, our results showing that inhibition of caspase-4 by Z-LEVD-FMK leading to inhibition of ZnO-induced production of IL-1 β are probably the first in this area. Furthermore, they are in agreement with the role of caspase-4 in regulating caspase-1 in humans, a process not observed in laboratory rodents (Kajiwaru et al., 2014). Of note, using another type of NP, nanosilver, we previously documented that this same caspase-4 inhibitor was also able to reverse the nanosilver-induced IL-1 β production in human neutrophils (Liz et al., 2015). Interestingly, our present results indicating that caspase-4 inhibitor can reverse the production of IL-8 but that caspase-1 inhibitor had no effect, suggest that caspase-4 is also involved in the production of IL-8 by human eosinophils, but the mechanism needs to be determined. However, in contrast, we previously demonstrated that arsenic trioxide-induced IL-8 production was not inhibited in the presence of Z-LEVD-FMK inhibitor in human neutrophils; it was even found to increase the effect of arsenic

trioxide (Binet et al., 2011). Therefore, based on these results, it is tempting to speculate that caspase-4 possesses differential roles in human eosinophils and neutrophils. This remains to be elucidated. Interestingly, in one study, caspase-4-deficient human THP-1 cells were found to exhibit a marked reduction in the production of different cytokines, including not only IL-8, but also IL-1 β , in response to LPS, indicating that this caspase is involved in cytokine production (Lakshmanan and Porter, 2007). Recently, we also reported that caspase-4 was involved in cytokine production since nanosilver-induced IL-1 β cytokine production was reversed by the Z-LEVD-FMK inhibitor, as well as the caspase-1 activity (Liz et al., 2015). Knowing now that ZnO NPs can increase the production of IL-1 β and IL-8 cytokines (*this report*), it will be important in future to determine if these NPs can increase the production of other cytokines and chemokines in order to better understand how they can affect not only the biology of human eosinophils but also other cells.

5. Conclusion

This is probably the first study showing that human eosinophils are cellular targets to nanoparticles, namely, ZnO. Also, we bring evidences that ZnO delay human eosinophil apoptosis by preventing caspase activation as well as caspase-4 and Bcl-xL degradation. This ability to delay eosinophil apoptosis has to be considered in terms of inflammatory potential. Indeed, increasing cell survival of eosinophils under certain circumstances could be deleterious for individuals. Therefore, the unique antiapoptotic activity of naked ZnO on human granulocytes, including eosinophils (*this report*) and neutrophils (Goncalves and Girard, 2014) has to be considered, especially when the aim is to use ZnO as potential drug delivery agents for the development of new therapeutic strategies (Xiong, 2013; Zhang et al., 2013). Similarly, the capacity of ZnO to increase the production of the proinflammatory cytokines IL-1 β and IL-8 by eosinophils should not be neglected.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgement

The study was supported by grants from the Institut de recherche Robert-Sauvé en santé et en sécurité du travail (IRSST).

References

- Akhtar, M.J., Ahamed, M., Kumar, S., Khan, M.M., Ahmad, J., Alrokayan, S.A., 2012. Zinc oxide nanoparticles selectively induce apoptosis in human cancer cells through reactive oxygen species. *Int. J. Nanomed.* 7, 845–857.
- Babin, K., Antoine, F., Goncalves, D.M., Girard, D., 2013. TiO₂, CeO₂ and ZnO nanoparticles and modulation of the degranulation process in human neutrophils. *Toxicol. Lett.* 221, 57–63.
- Barnes, P.J., 1996. Mechanisms of action of glucocorticoids in asthma. *Am. J. Respir. Crit. Care Med.* 154, S26–27.
- Binet, F., Cavalli, H., Moisan, E., Girard, D., 2006. Arsenic trioxide (AT) is a novel human neutrophil pro-apoptotic agent: effects of catalase on AT-induced apoptosis, degradation of cytoskeletal proteins and de novo protein synthesis. *Br. J. Haematol.* 132, 349–358.
- Binet, F., Chiasson, S., Girard, D., 2011. Interaction between arsenic trioxide (ATO) and human neutrophils. *Hum. Exp. Toxicol.* 30, 416–424.
- Braun, R.K., Franchini, M., Erard, F., Rihs, S., De Vries, I.J., Blaser, K., Hansel, T.T., Walker, C., 1993. Human peripheral blood eosinophils produce and release interleukin-8 on stimulation with calcium ionophore. *Eur. J. Immunol.* 23, 956–960.
- Chen, R., Huo, L., Shi, X., Bai, R., Zhang, Z., Zhao, Y., Chang, Y., Chen, C., 2014. Endoplasmic reticulum stress induced by zinc oxide nanoparticles is an earlier biomarker for nanotoxicological evaluation. *ACS Nano* 8, 2562–2574.
- De Berardis, B., Civitelli, G., Condello, M., Lista, P., Pozzi, R., Arancia, G., Meschini, S., 2010. Exposure to ZnO nanoparticles induces oxidative stress and cytotoxicity in human colon carcinoma cells. *Toxicol. Appl. Pharmacol.* 246, 116–127.
- Dibbert, B., Daigle, I., Braun, D., Schranz, C., Weber, M., Blaser, K., Zangemeister-Wittke, U., Akbar, A.N., Simon, H.U., 1998. Role for Bcl-xL in delayed eosinophil apoptosis mediated by granulocyte-macrophage colony-stimulating factor and interleukin-5. *Blood* 92, 778–783.
- Drulhe, A., Letuve, S., Pretolani, M., 2003. Glucocorticoid-induced apoptosis in human eosinophils: mechanisms of action. *Apoptosis* 8, 481–495.
- Esnault, S., Kelly, E.A., Nottenstrom, L.M., Cook, E.B., Seroogy, C.M., Jarjour, N.N., 2012. Human eosinophils release IL-1ss and increase expression of IL-17A in activated CD4+ T lymphocytes. *Clin. Exp. Allergy* 42, 1756–1764.
- Girard, D., Paquin, R., Beaulieu, A.D., 1997. Responsiveness of human neutrophils to interleukin-4: induction of cytoskeletal rearrangements, de novo protein synthesis and delay of apoptosis. *Biochem. J.* 325 (Pt 1), 147–153.
- Goncalves, D.M., Girard, D., 2014. Zinc oxide nanoparticles delay human neutrophil apoptosis by a de novo protein synthesis-dependent and reactive oxygen species-independent mechanism. *Toxicol. In Vitro* 28, 926–931.
- Goncalves, D.M., Chiasson, S., Girard, D., 2010. Activation of human neutrophils by titanium dioxide (TiO₂) nanoparticles. *Toxicol. In Vitro* 24, 1002–1008.
- Huang, K.L., Lee, Y.H., Chen, H.L., Liao, H.S., Chiang, B.L., Cheng, T.J., 2015. Zinc oxide nanoparticles induce eosinophilic airway inflammation in mice. *J. Hazard. Mater.* 297, 304–312.
- Ilmarinen, P., Kankaanranta, H., 2014. Eosinophil apoptosis as a therapeutic target in allergic asthma. *Basic Clin. Pharmacol. Toxicol.* 114, 109–117.
- Ilmarinen, P., Hasala, H., Sareila, O., Moilanen, E., Kankaanranta, H., 2009. Bacterial DNA delays human eosinophil apoptosis. *Pulm. Pharmacol. Ther.* 22, 167–176.
- Ilmarinen, P., Moilanen, E., Kankaanranta, H., 2014. Regulation of spontaneous eosinophil apoptosis—a neglected area of importance. *J. Cell Death* 7, 1–9.
- Kajiwar, Y., Schiff, T., Voloudakis, G., Gama Sosa, M.A., Elder, G., Bozdagi, O., Buxbaum, J.D., 2014. A critical role for human caspase-4 in endotoxin sensitivity. *J. Immunol.* 193, 335–343.
- Kim, M.H., Seo, J.H., Kim, H.M., Jeong, H.J., 2014. Zinc oxide nanoparticles, a novel candidate for the treatment of allergic inflammatory diseases. *Eur. J. Pharmacol.* 738, 31–39.
- Kovacs, I., Horvath, M., Kovacs, T., Somogyi, K., Tretter, L., Geiszt, M., Petheo, G.L., 2014. Comparison of proton channel, phagocyte oxidase, and respiratory burst levels between human eosinophil and neutrophil granulocytes. *Free Radic. Res.* 48, 1190–1199.
- Lakshmanan, U., Porter, A.G., 2007. Caspase-4 interacts with TNF receptor-associated factor 6 and mediates lipopolysaccharide-induced NF-kappaB-dependent production of IL-8 and CC chemokine ligand 4 (macrophage-inflammatory protein-1). *J. Immunol.* 179, 8480–8490.
- Lavastre, V., Chiasson, S., Cavalli, H., Girard, D., 2005. Viscum album agglutinin-I induces apoptosis and degradation of cytoskeletal proteins via caspases in human leukaemia eosinophil AML14.3D10 cells: differences with purified human eosinophils. *Br. J. Haematol.* 130, 527–535.
- Liz, R., Simard, J.C., Leonardi, L.B., Girard, D., 2015. Silver nanoparticles rapidly induce atypical human neutrophil cell death by a process involving inflammatory caspases and reactive oxygen species and induce neutrophil extracellular traps release upon cell adhesion. *Int. Immunopharmacol.* 28, 616–625.
- Man, S.M., Kanneganti, T.D., 2015. Regulation of inflammasome activation. *Immunol. Rev.* 265, 6–21.
- Moisan, E., Kouassi, E., Girard, D., 2003. Mechanisms involved in methylmercuric chloride (MeHgCl)-induced suppression of human neutrophil apoptosis. *Hum. Exp. Toxicol.* 22, 629–637.
- Onoue, S., Yamada, S., Chan, H.K., 2014. Nanodrugs: pharmacokinetics and safety. *Int. J. Nanomed.* 9, 1025–1037.
- Orazizadeh, M., Khodadadi, A., Bayati, V., Saremy, S., Farasat, M., Khorsandi, L., 2015. In vitro toxic effects of zinc oxide nanoparticles on rat adipose tissue-derived mesenchymal stem cells. *Cell J.* 17, 412–421.
- Poirier, M., Simard, J.C., Antoine, F., Girard, D., 2014. Interaction between silver nanoparticles of 20 nm (AgNP20) and human neutrophils: induction of apoptosis and inhibition of de novo protein synthesis by AgNP20 aggregates. *J. Appl. Toxicol.* 34, 404–412.
- Roy, R., Kumar, S., Verma, A.K., Sharma, A., Chaudhari, B.P., Tripathi, A., Das, M., Dwivedi, P.D., 2013. Zinc oxide nanoparticles provide an adjuvant effect to ovalbumin via a Th2 response in Balb/c mice. *Int. Immunol.* 13, 13.
- Shamri, R., Xenakis, J.J., Spencer, L.A., 2011. Eosinophils in innate immunity: an evolving story. *Cell Tissue Res.* 343, 57–83.
- Sharma, V., Anderson, D., Dhawan, A., 2012. Zinc oxide nanoparticles induce oxidative DNA damage and ROS-triggered mitochondria mediated apoptosis in human liver cells (HepG2). *Apoptosis* 17, 852–870.
- Shoae-Hagh, P., Rahimifard, M., Navaei-Nigeh, M., Baeri, M., Gholami, M., Mohammadirad, A., Abdollahi, M., 2014. Zinc oxide nanoparticles reduce apoptosis and oxidative stress values in isolated rat pancreatic islets. *Biol. Trace Elem. Res.* 162, 262–269.
- Simard, J.C., Simon, M.M., Tessier, P.A., Girard, D., 2011. Damage-associated molecular pattern S100A9 increases bactericidal activity of human neutrophils by enhancing phagocytosis. *J. Immunol.* 186, 3622–3631.
- Song, W., Zhang, J., Guo, J., Ding, F., Li, L., Sun, Z., 2010. Role of the dissolved zinc ion and reactive oxygen species in cytotoxicity of ZnO nanoparticles. *Toxicol. Lett.* 199, 389–397.
- Sruthi, S., Mohanan, P.V., 2015. Investigation on cellular interactions of astrocytes with zinc oxide nanoparticles using rat C6 cell lines. *Colloids Surf. B Biointerfaces* 133, 1–11.

- Stringer, R.E., Hart, C.A., Edwards, S.W., 1996. Sodium butyrate delays neutrophil apoptosis: role of protein biosynthesis in neutrophil survival. *Br. J. Haematol.* 92, 169–175.
- Suzuki, H., Toyooka, T., Ibuki, Y., 2007. Simple and easy method to evaluate uptake potential of nanoparticles in mammalian cells using a flow cytometric light scatter analysis. *Environ. Sci. Technol.* 41, 3018–3024.
- Szelenyi, I., 2012. Nanomedicine: evolutionary and revolutionary developments in the treatment of certain inflammatory diseases. *Inflamm. Res.* 61, 1–9.
- Tai, P.C., Sun, L., Spry, C.J., 1991. Effects of IL-5, granulocyte/macrophage colony-stimulating factor (GM-CSF) and IL-3 on the survival of human blood eosinophils in vitro. *Clin. Exp. Immunol.* 85, 312–316.
- Tran, H.B., Lewis, M.D., Tan, L.W., Lester, S.E., Baker, L.M., Ng, J., Hamilton-Bruce, M. A., Hill, C.L., Koblar, S.A., Rischmueller, M., Ruffin, R.E., Wormald, P.J., Zalewski, P. D., Lang, C.J., 2012. Immunolocalization of NLRP3 inflammasome in normal murine airway epithelium and changes following induction of ovalbumin-induced airway inflammation. *J. Allergy* 2012, 819176.
- Vigano, E., Diamond, C.E., Spreafico, R., Balachander, A., Sobota, R.M., Mortellaro, A., 2015. Human caspase-4 and caspase-5 regulate the one-step non-canonical inflammasome activation in monocytes. *Nat. Commun.* 6, 8761.
- Wahab, R., Siddiqui, M.A., Saquib, Q., Dwivedi, S., Ahmad, J., Musarrat, J., Al-Khedhairi, A.A., Shin, H.S., 2014. ZnO nanoparticles induced oxidative stress and apoptosis in HepG2 and MCF-7 cancer cells and their antibacterial activity. *Colloids Surf. B Biointerfaces* 117, 267–276.
- Wang, J., Deng, X., Zhang, F., Chen, D., Ding, W., 2014. ZnO nanoparticle-induced oxidative stress triggers apoptosis by activating JNK signaling pathway in cultured primary astrocytes. *Nanoscale Res. Lett.* 9, 117.
- Wang, C., Hu, X., Gao, Y., Ji, Y., 2015. ZnO nanoparticles treatment induces apoptosis by increasing intracellular ROS levels in LTP-a-2Cells. *BioMed Res. Int.* 2015, 423287.
- Wong, C.K., Dong, J., Lam, C.W., 2014. Molecular mechanisms regulating the synergism between IL-32 gamma and NOD for the activation of eosinophils. *J. Leukoc. Biol.* 95, 631–642.
- Wu, W., Samet, J.M., Peden, D.B., Bromberg, P.A., 2010. Phosphorylation of p65 is required for zinc oxide nanoparticle-induced interleukin 8 expression in human bronchial epithelial cells. *Environ. Health Perspect.* 118, 982–987.
- Xiong, H.M., 2013. ZnO nanoparticles applied to bioimaging and drug delivery. *Adv. Mater. (Deerfield Beach, Fla.)* 25, 5329–5335.
- Zhang, Y., Nayak, T.R., Hong, H., Cai, W., 2013. Biomedical applications of zinc oxide nanomaterials. *Curr. Mol. Med.* 13, 1633–1645.