



Activation of Human Eosinophils with Nanoparticles: a New Area of Research

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Abstract— It is becoming increasingly clear that nanoparticles (NPs) possess many potential applications in both clinical medicine and research. Potential utilization of NPs in nanomedicine for the treatment of respiratory diseases where eosinophils exert pathogenic roles is gaining increasing attention. Even though several NPs were found to possess pro-inflammatory activities in *in vivo* models based on an increased number of eosinophils in rodent airways, it is not clear how NPs could directly activate eosinophils themselves and how they can alter their biology. In this review, we discuss the most recent data in this new area of research demonstrating that NPs could now be added as new eosinophils modulators. Indeed, activation of eosinophils with NPs could lead to modulation of spontaneous apoptosis, caspase activation, and cytoskeleton breakdown when apoptosis is induced; cytokine production, *de novo* protein synthesis, cellular adhesion onto a cell substratum, and cell signaling events such as activation of the phosphoinositide 3-kinase/Akt pathway and actin re-localization are involved in NP-induced adhesion. Therefore, future development of therapeutic strategies with NPs aiming at targeting diseases where eosinophils are involved should now consider the capacity of NPs to modulate human eosinophil biology.

KEY WORDS: nanoparticles; inflammation; eosinophils; apoptosis; cytoskeleton; cytokines; cellular adhesion.

INTRODUCTION

Granulocytes

Granulocytes are a subtype of leukocytes characterized by the presence of several granules in their cytoplasm.

Using a combination of acidic (eosin) and basic (methylene blue) dyes like in the May-Grünwald Giemsa staining, the granules, as well as nuclei and cytoplasm, will be differentially stained leading to the distinction of three types of granulocytes, neutrophils, basophils, and eosinophils. While the cytoplasm of neutrophils is stained light pink, their granules will be colored as light-pink to bluish-purple (“neutral”). In basophils, their large granules will appear deep blue to violet (“baso”), whereas the granules in eosinophils will be stained orange/red (*eosin*). All three types of granulocytes are well known for their role in inflammatory conditions. In human, under normal conditions, neutrophils are the most abundant leukocytes in the blood representing up to 50–70% of these cells, whereas basophils and eosinophils account for less than 1% and ~3%, respectively. This review will focus strictly on granulocyte

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eosinophils and will cover how nanoparticles can alter their biology, a new area of research.

Eosinophils

Eosinophils originated from the bone marrow and are derived from CD34⁺ stem cells. The progenitor cells respond to the cytokines interleukin (IL)-3, IL-5, and granulocyte-macrophage colony-stimulating factor (GM-CSF), and IL-5 is particularly known as a strong and specific inducer of eosinophil differentiation. In fact, committed eosinophils progenitors are designed as CD34⁺IL-5R⁺ and the cell turnover of eosinophils was estimated at $\sim 2 \times 10^8$ cells/kg/day. When cells are mature, they are then released from the bone marrow to the bloodstream. Interestingly, eosinophils are not abundant in the circulation and this could be explained by their sort half-life of less than ~ 18 h and by their ability to undergo spontaneous apoptosis, a very important biological process controlling the number of eosinophils [1–3]. When they are isolated from the blood of healthy individuals, eosinophils will spontaneously undergo apoptosis when incubated *in vitro* in absence of any modulators of apoptosis. In such conditions, more than $\sim 50\%$ of eosinophils will be apoptotic within 2 days of culture [3].

Eosinophils are well known for their role in the host defense to fight against parasitic pathogens and to exert pivotal roles in the pathogenesis of asthma and allergic diseases [4, 5]. In fact, the list of human diseases in which eosinophils can play a pathologic role is vast and also include diseases like Wegener's granulomatosis, multiple sclerosis, sarcoidosis, and idiopathic pulmonary fibrosis, to name a few [1]. The implication of eosinophils in inflammatory conditions of the skin, gastrointestinal tract, and airways is well documented. For example, an increased number of eosinophils are not only observed in the blood or sputum of patients suffering from eosinophilic asthma, but it also correlates with disease severity [6, 7]. Of note, although all types of leukocytes could be present in airway inflammatory infiltrates, more than 50% of cells are typically eosinophils [8]. Increasing evidence revealed additional roles for eosinophils in regulating immune responses and inflammation, especially *via* their ability to secrete different mediators, including a variety of cytokines and chemokines such as transforming growth factor beta (TGF- β), IL-1 β , IL-3, IL-5, IL-8 (CXCL8), and IL-13 [4, 6, 9, 10]. As other leukocytes, upon certain inflammatory conditions, eosinophils will leave the blood circulation by starting rolling onto endothelial cells forming the blood vessels and then will tether to initially adhere slightly *via*

interaction with selectin receptors and then more firmly *via* the interaction with integrin receptors [11]. After, they must migrate through endothelial cells to leave the blood vessel and migrate to, for example, an inflammatory site. Figure 1 illustrates this biological process. To prevent accumulation of eosinophils and unregulated inflammatory response, cells must be properly eliminated, and this occurs by falling into an apoptotic state.

Apoptotic eosinophils are known to be eliminated by professional phagocytes, including resident or recruited monocytes-macrophages as well as by “non-professional” phagocytes such as epithelial cells *via* efferocytosis (Fig. 2), a crucial process for the resolution of the airway inflammation [12, 13]. In fact, defects in apoptosis and/or subsequent efferocytosis of inflammatory cells are known to be involved in chronic inflammatory diseases of the lung and autoimmune diseases [7, 14]. Several agents are known to prolong eosinophil survival by delaying apoptosis, including the cytokines GM-CSF, IL-3, and IL-5 [3, 15, 16]. Interestingly, these cytokines are present in the lungs of asthmatic patients [3, 15]. In contrast, other agents can induce or accelerate eosinophil apoptosis, including hydrogen peroxide [17], the plant lectin *Viscum album* agglutinin-1 [18], and glucocorticoids [19]. Glucocorticoids are known to be effective for the treatment of eosinophilic disorders such as asthma [20]. Therefore, identification of new agents having the capacity to modulate human eosinophil apoptosis and/or efferocytosis represents an important step for the development or improvement of therapeutic strategies targeting eosinophils.

Nanoparticles

Nanoparticles (NPs) are small-scale substances with at least one dimension < 100 nm. They possess an increased surface area and small size over their corresponding bulk materials given them, for example, unique mechanical, optical, electrical, and magnetic properties. Although engineered NPs are recently increasingly used in a variety of sectors, including electronic, cosmetics, textile, and even have in food industry, large quantities of NPs are released into the environment from natural sources such as fires, volcanic eruptions, oceans, and groundwater for many years. Of note, the behaviors of NPs in the environment and their interactions with biological systems are still largely unknown.

In the past few years, an interest to use NPs for medical diagnosis and therapeutic purposes has rapidly increased. The potential use of NPs for drug delivery in

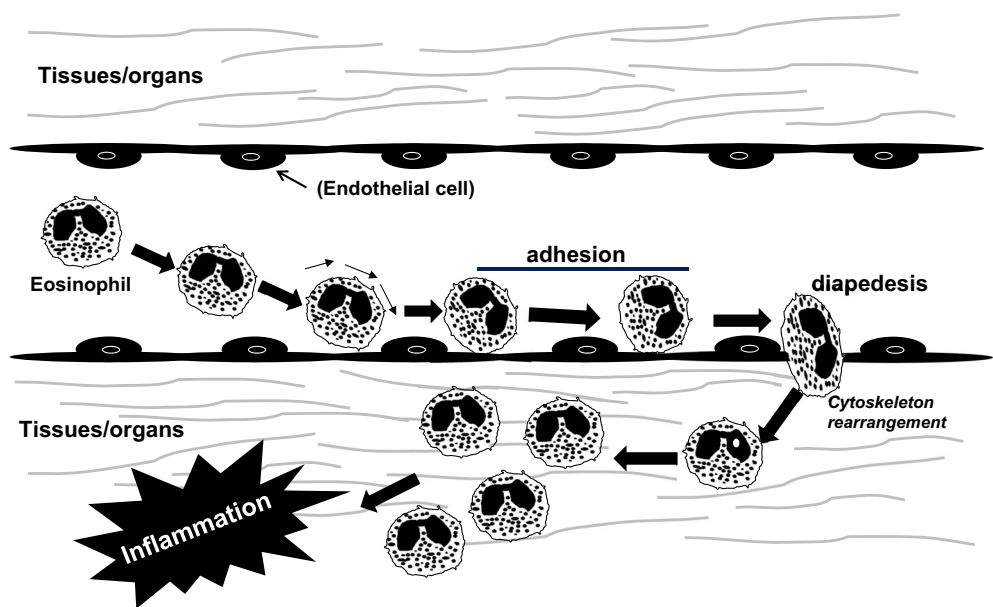


Fig. 1. Illustration showing how eosinophils leave the blood circulation to migrate to an inflammatory site. Under appropriate conditions (allergens or other inflammatory stimuli), eosinophils will be activated/attracted to leave the blood circulation. They will initially start to roll onto endothelial cells and adhere onto them slightly and then more firmly. Next, they will transmigrate *via* diapedesis where their cytoskeleton will be rearranged and cells will, at the end, readopt their initial shape. If not eliminated properly, eosinophils will accumulate and cause inflammation.

cancer therapies is very attractive [21, 22]. Also, other studies promote the use of NPs as carriers to detect allergies or to alleviate inflammatory symptoms [23, 24]. Despite

the interest to use NPs for medical purposes, there are some potential toxic risks in human since, cytotoxicity, oxidative stress, genotoxicity, and inflammation have been reported,

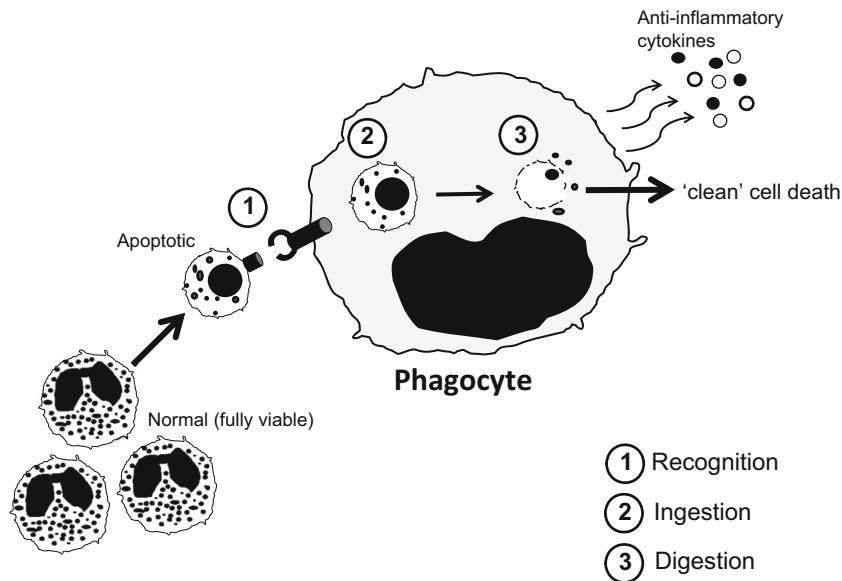


Fig. 2. Illustration of efferocytosis, a mechanism involved in the resolution of inflammation. Efferocytosis is exerted by professional phagocytes and consist in three major steps: recognition (1), ingestion (2), and digestion of apoptotic cells like eosinophils (3). In addition, phagocytes will release anti-inflammatory mediators such as cytokines. The overall process prevents accumulation of eosinophils that could lead to inflammation.

both in a variety of *in vitro* and *in vivo* models [25]. *In vitro*, the cytotoxicity of different types of NPs has been largely documented using a variety of cell lines of different origins [26–32]. In these studies, several kinetics and dose-response experiments have been reported, making direct comparisons between the available studies difficult. Of note, most studies used cancerous transformed cells and not normal primary cells. We are among few laboratories investigating the toxic effects and mode of action of NPs on primary human cells, especially human granulocytes such as neutrophils [33–40], largely because we are interested in determining the inflammatory properties of NPs. Also, it is curious to note in the literature that the number of granulocytes is frequently simply used as a marker of inflammation in diverse NP-induced models of inflammation; the higher the number of cells, the more the NP is considered as being pro-inflammatory. Before our works in this area, there were very studies reporting the direct effect of NPs on the biology of human granulocytes. More recently, we initiated studies aiming at determining how NPs could alter the biology of the neutrophil's sister cell, the eosinophil, the object of this review.

Interaction NP-Eosinophils

Activation of Eosinophil-Like Cells

The characteristics of the AML14.3D10 cell line and how it has been used to obtain important information relevant to human eosinophil biology have been reported more than 20 years ago [41]. These cells are a subclone of the AML14 cell line that had grown continuously in culture for several months. This AML14 human myeloid leukemia cell line was established several years ago, in 1992. They were obtained from a 68-year-old man suffering from an acute myeloid leukemia. They have been subcloned by limiting dilution and the subclone that both proliferated vigorously and maintained an advanced eosinophilic phenotype was selected for further study. It was named AML14.3D10 because it grew out of the D10 well of the third 96-well plate used for the cloning. Interestingly, this subclone was found to maintain an advanced eosinophil phenotype and to proliferate very well by a cytokine-independent fashion. Since then, the utilization of AML14.3D10 cells has been reported in several studies in which they were used as a good laboratory tool for studying eosinophil cell biology and/or to get information on cancerous cells of eosinophilic origin [42–45]. In the past few years, we have used this cell line in order to study how they will respond to a direct activation with a panel of NPs, including titanium dioxide (TiO₂), cerium oxide

(CeO₂), zinc oxide (ZnO), as well as nanosilver of initial diameter of 20 nm (AgNP₂₀) or 70 nm (AgNP₇₀) [46]. At a non-necrotic concentration of 100 µg/ml, only ZnO and AgNP₂₀ were found to be pro-apoptotic for AML14.3D10 cells, albeit less than the potent pro-apoptotic plant lectin *Viscum album* agglutinin-I (VAA-I). Interestingly, unlike VAA-I that was found to activate caspase-3, caspase-7, and caspase-9, AgNP₂₀ did activate caspase-3 and caspase-7 but not caspase-9 while ZnO did not activate these caspases like the other tested NPs that were not pro-apoptotic. However, as for VAA-I, AgNP₂₀ and ZnO were the only tested NP found to induce cleavage of lamin B1, as judged by a decrease expression of the mature protein and an increase expression of the cleaved lamin B1 fragment, indicating cytoskeleton breakdown normally observed during cell apoptosis. Figure 3 summarizes these events. In addition, although some NPs increased the expression of different cytokines and chemokines, AgNP₂₀ and ZnO were the most potent ones, especially for the IL-8 production. Collectively, these results indicate that NPs can activate human cells of eosinophilic origin at least regarding their modulatory ability on apoptosis and cytokine production. Of note, AML14.3D10 cells respond differently to diverse NPs and the mode of action of NPs appears to be different among the tested NPs as judged, for example, by the ability of both AgNP₂₀ and ZnO to induce apoptosis and based on their different capacities to activate caspases.

Activation of Primary Human Eosinophils, the First Evidences That These Cells Are Targets to NPs

The above results suggest that eosinophils represent potential targets to NPs and therefore prompted us to study the direct interaction between primary human eosinophils and NPs. To do so, human eosinophils were freshly isolated from healthy volunteers by a negative selection strategy using magnetic CD16 beads targeting neutrophils during granulocyte isolation [18]. Figure 4 illustrates the granulocyte cell preparation (mainly neutrophils and few eosinophils) obtained after human blood granulocyte cell isolation (Fig. 4a) and the eosinophil enriched cell population after their separation from neutrophils by negative immunomagnetic selection using anti-human CD16-coated magnetic beads. In Fig. 4c, apoptotic eosinophils (filled arrows) could be easily identified when compared with normal eosinophils harboring a typical bilobed nucleus (open arrows). In the first study, we investigate the interaction between ZnO NPs and eosinophils, especially because conflicting

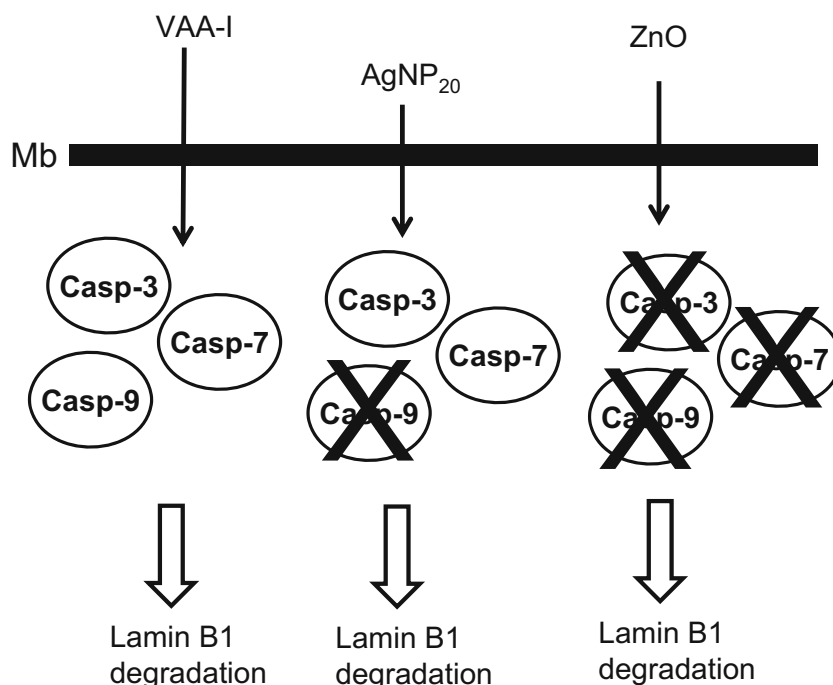


Fig. 3. Illustration showing how two different NPs can differently lead to the degradation of lamin B1 in human eosinophils. The plant lectin *Viscum album* agglutinin-I (VAA-I), the nanoparticles AgNP₂₀, and ZnO lead to the degradation of lamin B1 but by activating different caspases.

results were reported. Indeed, not only ZnO were proposed as interesting potential candidates for the treatment of allergic inflammatory diseases [47], but also these NPs were reported to induce eosinophilic airway inflammation *in vivo* [48]. In addition to these paradoxical observations, the fact that we demonstrated that ZnO could also modulate AML-14.3D10 cells further prompted us to study how ZnO could alter the biology of human eosinophils [49]. In this very first study reporting that human eosinophils are in fact direct targets to NPs, we found that in contrast to their pro-apoptotic activity observed in AML14.3D10 cells, ZnO were found to delay eosinophil apoptosis. However, in both cases, ZnO induced the production of IL-8. Also, in this study, caspase inhibition was among the mechanism involved in ZnO-induced suppression of apoptosis. Interestingly, ZnO were found to induce *de novo* protein synthesis but not ROS production in eosinophils, suggesting that some newly synthesized polypeptides are involved in the ability of ZnO to delay apoptosis. This was also observed previously when we investigated how ZnO alter the biology of human neutrophils. It remains to be determined if the same newly synthesized proteins are involved on both cell types in response to ZnO.

NPs Can Increase Adhesion of Human Eosinophils onto Endothelial Cells

As illustrated in Fig. 1, adhesion of eosinophils onto endothelial cells represents one important step for eosinophils to leave the bloodstream and reach, for example, airways after appropriate antigen stimulation [11]. Therefore, we investigated how NPs could alter cellular adhesion of human eosinophils onto human umbilical vein cell line Ea.hy926 cells [50]. Eosinophils were stained with calcein-AM, treated with NPs, incubated onto endothelial cells, and washed, and the number of adherent cells was counted. By testing a panel of ten different NPs at the same concentration of 50 µg/ml, all, but one (AuNP⁻), were found to increase cellular adhesion from indices ranging from ~1.3 to up to ~3, TiO₂ NPs, being the most potent. In order to determine the mode of action, we next focus on TiO₂ where a clear concentration-response effect was observed. Indeed, the index was increased to more than 7 when tested at 150 µg/ml, indicating that high concentration of NPs could markedly increase the adhesion of human eosinophils onto a cell substratum, like endothelial

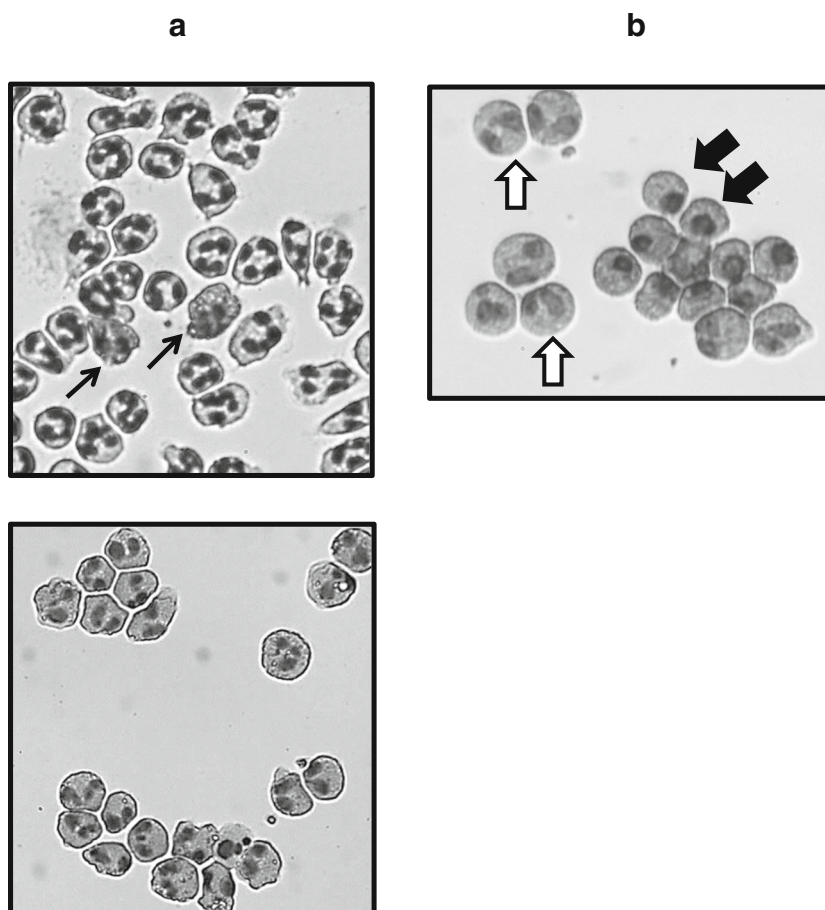


Fig. 4. Images of human granulocytes. The upper part of **a** illustrates a typical cell preparation of human granulocytes containing mainly neutrophils and few eosinophils (arrows). After the negative selection strategy using magnetic CD16 beads to eliminate neutrophils, an enriched population of eosinophils is obtained (**a**, bottom part). **b** Illustration of normal eosinophils with typical bilobed nuclei (white arrows) and apoptotic eosinophils (black arrows) during spontaneous apoptosis observed after 48 h.

cells. The effect of TiO_2 was found to be independent on increased expression of several cell surface adhesion molecules expressed on eosinophils, including ICAM-1, ICAM-3, CD11a, CD11b, CD18, CD29, CD49d, and CD62L. In fact, the cell surface expression remains similar to that on untreated cells, except for CD29 that was even found to be significantly decreased [50]. However, we demonstrated that TiO_2 NPs rapidly induced Akt phosphorylation (but not Erk-1/2) and pre-treatment of eosinophils with the PI3K/Akt inhibitor wortmannin was found to not only reverse phosphorylation of Akt, but also it drastically inhibits TiO_2 -induced cellular adhesion (see Fig. 5 for a summary).

NPs Can Target the Eosinophil Cytoskeleton

Based on the fact that some NPs could induce eosinophil apoptosis and their adhesion onto endothelial cells, two important functions requiring cytoskeleton rearrangement, we next investigate the importance of the actin cytoskeleton in Pd-NP-induced adhesion onto Ea.hy926 cells. In addition, previous data obtained in AML14.3D10 eosinophilic cell line, demonstrating that some cytoskeleton proteins are degraded during NP-induced apoptosis [46], was also taken into account to investigate the role of the cytoskeleton in NP-induced adhesion of eosinophils. Using AML14.3D10 cells, we have shown that Pd-NPs, at non necrotic concentrations, did not induce apoptosis [51].

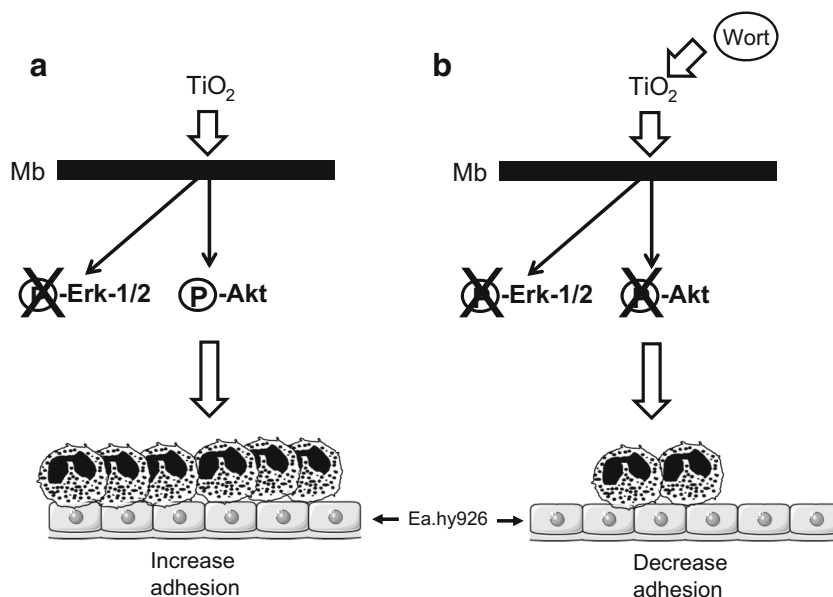


Fig. 5. Illustration showing that TiO₂ NPs can increase adhesion of human eosinophils onto endothelial cells by an Akt-dependent mechanism. TiO₂ NPs induce phosphorylation of Akt (p-Akt) but not Erk-1/2 and increase adhesion of eosinophils onto endothelial EA.hy926 cells (a), but when cells are preincubated with the inhibitor wortmannin (wort), Akt is dephosphorylated and the number of adherent eosinophils drastically decreased (b).

Actin re-localization was observed by immunofluorescence after treatment of cells with Pd-NPs, indicating cytoskeleton reorganization. Pd-NPs were found to increase adhesion of AML-14.3D10 cells onto endothelial cells, and the use of the cell-permeable and potent inhibitor of actin polymerization, cytochalasin D, drastically reversed the effect of Pd-NPs. Using freshly isolated human eosinophils, Pd-NPs were also found to increase their adhesion onto endothelial cells and this was reversed using cytochalasin D, demonstrating the importance of the actin cytoskeleton in Pd-NP-induced eosinophil adhesion [51].

CONCLUSIONS

Taken all together, the *in vivo* observations reported by different teams and, more specifically, our own studies dealing on the direct interaction of NPs with primary human eosinophils clearly indicated that these cells are targets to NPs. We are aware that the mode of action of NPs is far from being clear; in this review, we clearly indicate that ZnO NPs can delay human eosinophil apoptosis by suppressing caspases and by inducing *de novo* protein synthesis. They also increase cytokine production. Also, these NPs and several others, TiO₂ being the more

potent, can increase adhesion of eosinophils by an Akt-dependent mechanism. Also, NPs, such as Pd-NPs, can target the eosinophil cytoskeleton. We are also aware that the mode of action could differ from one NP to the other, increasing the complexity to study how they alter the biology of eosinophils.

Perspectives

In the future, accumulation of data emanating from studies investigating the direct effects of NPs on human eosinophil granulocyte biology will be of great importance for better predicting their safer use in clinical medicine.

We propose and encourage further investigations aiming at understanding how a given NP could alter the biology of human cells since this could even lead to unexpected discoveries. For example, using AgNPs, we identified a novel axis linking endoplasmic reticulum stress to inflammation *via* ATF6 (activating transcription factor 6) activation in human myeloid cells [52]. Indeed, a crucial role of ATF was demonstrated in caspase-1 processing and activation, secretion of IL-1 β , and pyroptosis induction. We believe that further investigating the direct effects of NPs on primary human cells will lead to yet, unexpected discoveries.

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