



# The effect of dust storm particles on single human lung cancer cells

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## ARTICLE INFO

### Keywords:

Dust storm particles  
Single cell analysis  
Cell death  
Cell division  
Engulfing

## ABSTRACT

Exposure to dust particles during dust storms can lead to respiratory problems, diseases, and even death. The effect of dust particles at the *cellular* level is poorly understood. In this study, we investigated the impact that dust storm particles (*Montmorillonite*) have on human lung epithelial cells (A549) at the single cell level. Using live-cell imaging, we continuously followed individual cells after exposure to a wide range of concentrations of dust particles. We monitored the growth trajectory of each cell including number and timing of divisions, interaction with the dust particles, as well as time and mechanism of cell death. We found that individual cells show different cellular fates (survival or death) even in response to the same dust concentration. Cells that died interacted with dust particles for longer times, and engulfed more dust particles, compared with surviving cells. While higher dust concentrations reduced viability in a dose-dependent manner, the effect on cell death was non-monotonic, with intermediate dust concentration leading to a larger fraction of dying cells compared to lower and higher concentrations. This non-monotonic relationship was explained by our findings that high dust concentrations inhibit cell proliferation. Using cellular morphological features, supported by immunoblots and proinflammatory cytokines, we determined that apoptosis is the dominant death mechanism at low dust concentrations, while higher dust concentrations activate necrosis. Similar single cell approaches can serve as a baseline for evaluating other aerosol types that will improve our understanding of the health-related consequences of exposure to dust storms.

## 1. Introduction

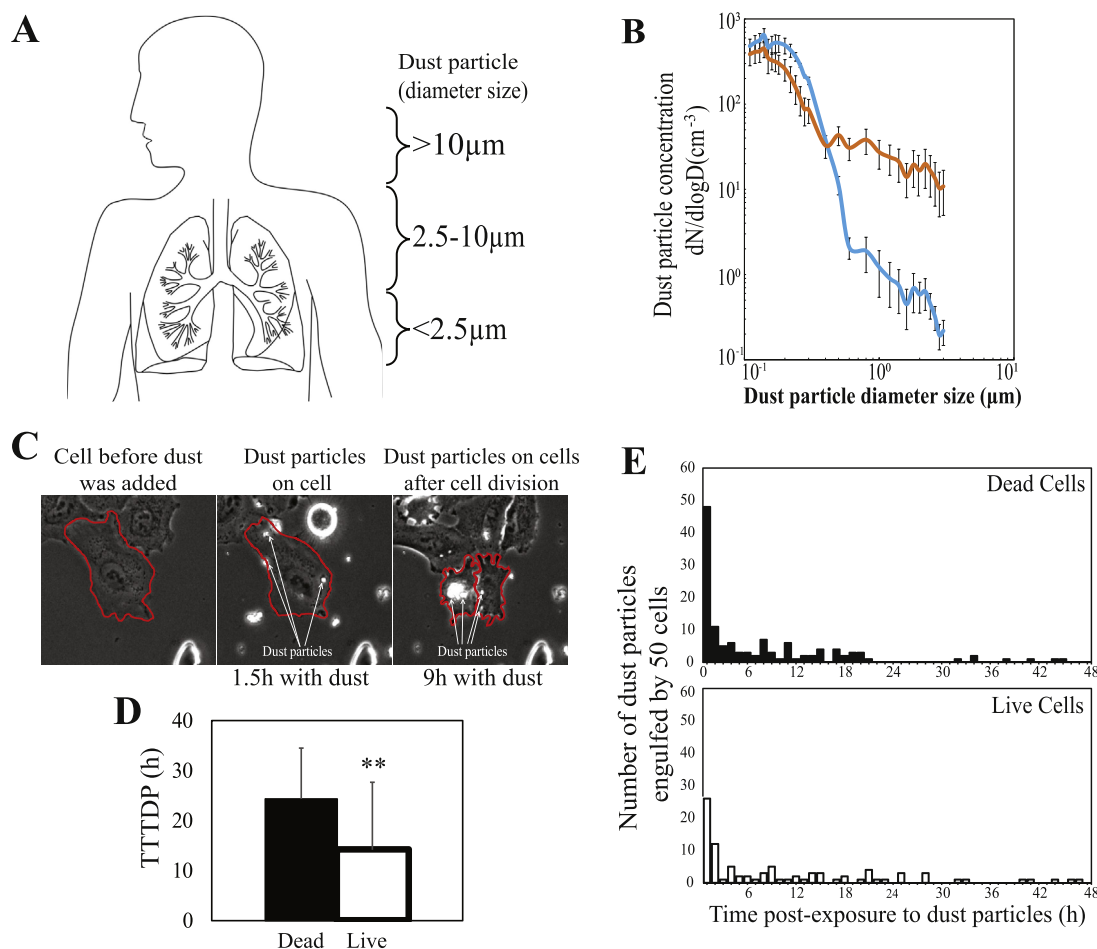
Aerosol particles (natural and anthropogenic) are an important, yet variable component of our atmosphere. Their type, size and concentration define air quality, and they can be suspended over long times and travel over long distances (Almeida-Silva et al., 2013; Kim et al., 2015). A large number of epidemiological studies demonstrated that large amounts of aerosol particles (Particulate Matter, PM) correlate with the number of daily deaths and hospitalizations, most likely as a result of respiratory and cardiovascular diseases (Peters, 2011; Karanasiou et al., 2012; Di et al., 2017). According to Cohen et al. (2004), PM are responsible for approximately 1.4% of deaths worldwide.

Dust storms are one of the most common natural aerosol particles sources. Strong dust storms can last from several hours to days (Vodonos et al., 2015; Crooks et al., 2016). Dust storm particles can transport particulate material, pollutants, and potential allergens (bioparticulates and microorganisms) over thousands of kilometers from

their source and were shown to reach locations far from the source. For example, dust from West Africa can cross the Atlantic to reach America (Almeida-Silva et al., 2013; Goudie, 2014). During a dust storm event, the particle concentrations often exceed the World Health Organization (WHO) health-recommended daily threshold values of PM<sub>10</sub> (particles with aerodynamic diameter < 10 μm) and PM<sub>2.5</sub> (particles with aerodynamic diameter < 2.5 μm) (Goudie and Middleton, 2006). Once inhaled, particles will rest in the respiratory tract according to their size (Fig. 1A). Generally, dust storm events are characterized by high concentration of coarse particles > 2.5 μm (Almeida-Silva et al., 2013). However, dust storms were shown to also contain high concentration of smaller particles. For example, in our previous work we found higher concentrations of small particles (0.5–3 μm) during dust storms in Israel compared to non-dust storm days (Fig. 1B, adapted from Ardon-Dryer and Levin (2014)). Particles of this size (< 2.5 μm) can penetrate the deepest part of the lungs (Goudie, 2014).

There is a growing concern regarding the impact of dust particles on human health. Dust particles that enter the lungs can cause local

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**Fig. 1.** Dust storm event contains small and breathable particles that effect cell viability due to engulfing. **(A)** Breathing particles as a function of the particle sizes. The location is dependent on the particle sizes, where small particles can penetrate deep into the lungs. **(B)** Higher concentration of breathable particles ( $0.5\text{--}3\mu\text{m}$ ) during dust storm. Average number concentration of particles in the size range  $0.11\text{--}3\mu\text{m}$ . The particles were measured during two dust storm days (orange) and during four clean days, non-dust storm days (blue), from Ardon-Dryer and Levin (2014) with adaption. Error bars represent standard error. **(C)** Cells touching dust particles at different time point. **(D)** TTTDP for 50 live cells (white column), which remain alive for 72 h and 50 cells which die during these 48 h (black column) at dust concentration of  $25\mu\text{g ml}^{-1}$ . Asterisks indicate significantly different between TTTDP of dead vs live cells (ANOVA).  $**P < 0.001$ . **(E)** Cells engulfed more particles in the first hour. Number of dust particles engulfed by live cells (lower row- white columns) or dead cells (upper row-black columns), as function of time (each column represents 1 h, first hour does not include the first image when dust added to the media) at dust concentration of  $25\mu\text{g ml}^{-1}$ .

damage as well as subsequent systemic damage elsewhere in the body, including the heart, skin and the brain (Goudie, 2014; Zhang et al., 2016). Dust particles that remain for several hours to days not only increase the incidence of respiratory issues (including asthma, tracheitis, pneumonia), but also cause other diseases, such as cardiovascular disorders stroke, conjunctivitis, skin irritations, meningococcal meningitis and valley fever (Goudie, 2014) and in rare cases can even result in death (Panikkath et al., 2013; Zhang et al., 2016). The mechanisms leading to health issues caused by the penetration of dust particles is still not fully understood (Deng et al., 2007; Yang et al., 2017).

Human lungs have multiple defense mechanisms for coping with large number of particles that result from exposure to high dust concentrations (Salvi and Holgate, 1999). Phagocytosis, for example, is an important mechanism for clearance of dust particles from the lungs by alveolar macrophages and epithelial cells (Jordan et al., 2009; Wang et al., 2009). Although the lungs can effectively cope with a large number of particles, these defense mechanisms are often overwhelmed by either the large number of particle or by the inherent toxicity of the particles (Salvi and Holgate, 1999). Above a certain particle

concentration, the cells phagocytic capacity decreases; cells become overloaded, and stop engulfing the particles (Bowden, 1987). Phagocytosis of particles by alveolar macrophages and epithelial cells were shown to lead to increased production and release of pro-inflammatory cytokines (Salvi and Holgate, 1999). This in turn can lead to asthma attack, respiratory syndromes and cardiovascular events. In patients compromised with lung functions, such as defective mucociliary clearance, or by abnormal immune function, even a small particle load may be sufficient to produce harmful effects on the lung (Salvi and Holgate, 1999).

Previous in vitro studies assessed the role of dust particles on human and mouse cells (Becher et al., 2001; Veranth et al., 2006; Yamada et al., 2012; Higashisaka et al., 2014; Pardo et al., 2017). These studies focused on how dust particles affect cell viability (cell death), which was represented by the percentage of cells that died after exposure to a specific dust concentration. It was also shown that an increase in dust concentrations leads to a decrease in cell viability (Veranth et al., 2004, 2006; Rodríguez-Cotto et al., 2013; Chang et al., 2016). Recent studies examined the effect of dust particles on live cells and discovered that an increase in dust concentrations inhibit cell proliferation (Deng et al.,

2007; Yang et al., 2017; Zeng et al., 2016). Note that these studies examined the exposure to dust at a single time point and in a bulk population of cells (Yamada et al., 2012; Ortiz-Martínez et al., 2015) masking the *dynamics* of cell growth and death in single cells and the *heterogeneity* between cells.

In this work, we examined the effect of dust particles on live human lung epithelial cells at the single cell level. We used live-cell imaging to collect quantitative measurements of individual cells in response to dust. We investigated the relationship between cellular death and the number of particles engulfed by cells as well as the dust effect on cell growth and cell death. Our study revealed cellular behaviors that were previously masked by averaging populations of cells or by looking at a few snapshots post exposure to dust.

## 2. Material and methods

### 2.1. Cell culture and cell line construction

The human lung epithelial cell line A549 was obtained from the NCI-Frederick Cancer DCTD Tumor/Cell Line Repository. Cells were grown as a monolayer and were maintained and treated in RPMI-1640 supplemented with 10% FBS, 2% L-glutamine and 1% penicillin/streptomycin and maintained in continuous culture at 37 °C with 5% CO<sub>2</sub>. Cells were infected with the lentiviral construct EF1a-mCerulean3-NLS, a constitutive fluorescent nuclear marker, which allowed to track individual cells after dust exposure.

### 2.2. Live-cell microscopy

Approximately 30,000 cells were plated 48 h prior to imaging on a poly-D-lysine-coated glass bottom dishes (MatTek). 24 h before imaging, media was replaced to RPMI without phenol red and riboflavin supplemented with 5% FBS. The cells were imaged on a Nikon Eclipse Ti inverted microscope in an enclosure to maintain humidity, a temperature of 37 °C and 5% CO<sub>2</sub>. Images were captured every 15-min using Meta Morph software.

Microscopy data were processed with a custom MATLAB code. Single-cell tracking was performed using the nuclear marker or phase images (Fig. S1). An open-source code used for this data analysis is available as part of the p53Cinema single cell analysis package at <https://github.com/balvahal/p53CinemaManual/releases>. Additional information on our single-cell tracking method can be found in Reyes et al. (2018).

### 2.3. Dust cells exposure

Montmorillonite particles, which are common in many dust storms worldwide (Ardon-Dryer and Levin, 2014; Engelbrecht et al., 2016), were used as model for dust storm particles. The particles were purchased from the Clay Mineral Society (SWy-2 Na-rich Montmorillonite from Wyoming, USA) and had a mode size of 2.5 μm. Various dust concentrations (10, 25, 50, 100, 250, 500 and 1000 μg ml<sup>-1</sup>) were made by adding a known mass concentration to fresh media following serial dilutions. Cells were imaged for 3 h before to exposing them to the media with dust.

### 2.4. Crystal violet and calculation of colonies number and sizes

100 cells were plated per well in a six well plate. After five days media was changed to media with dust at different concentrations. Six days after dust was added, colonies were stained with crystal violet (Franken et al., 2006). Each plate was scanned in a Nikon Eclipse Ti inverted microscope with a 2x magnification. Calculation of the number of surviving colonies (SF- surviving fraction) was based on Franken et al. (2006) and using ImageJ software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, <https://imagej.nih.gov/ij/>, 1997–2018. Based on <https://imagej.nih.gov/ij/docs/faq.html#cite>). Each colony was measured with a circle around its edges, representing the colony's area. Colonies were considered only when their area was larger than 658 μm<sup>2</sup>. This area represented a 29 μm colony diameter, below this size the colony shape was not well defined.

### 2.5. Cell division and cell death

Changes in the cell nuclei allowed identification and timing of cell division or cell death. Cell division was based on characteristic rounding up of the cell, followed by the appearance of two cells (as shown in Fig. S2). Cell death and time of death was based on differing the cell nuclei. While live cells maintained their nuclei shape throughout imaging (as shown Movie S1A), dead cells nuclei shape changed (as shown Movie S1B). Time of death was identified as the first time point in which the nuclei changed its shape before the cell disconnected from the surface. In the untreated condition case, a total of 700 cells were tracked. In each dust concentration 350 cells were tracked.

#### 2.5.1. Identifying cell death type

The mechanism of cell death was determined based on the cell nuclei shape. Examples of the two cell death mechanisms (apoptosis and necrosis) can be found in Movies S2A-B. Cells undergoing necrosis swell and lose their membrane integrity before releasing their intracellular contents into the surrounding environment. Cells undergoing apoptosis go through a series of well-defined events such as the shrinking of the cytoplasm, and cleavage into smaller fragments of apoptotic bodies without plasma membrane breakdown (Edinger and Thompson, 2004; Krysko et al., 2008; Nikolettou et al., 2013; Vanden Bergh et al., 2013).

### 2.6. Western blot analysis

$5 \times 10^5$  cells were incubated in media containing dust particles at different concentrations (no dust, 10–1000 μg ml<sup>-1</sup>) for 24 h. Cells were harvested, and protein samples were obtained by lysis. Equal protein amounts were separated by electrophoreses on 4%–12% Bis-Tris gradient gels (Invitrogen) and transferred to PVDF membranes by electroblotting. c-PARP was detected using monoclonal Y34 antibody (Abcam), actin using monoclonal AC-74 antibody (Sigma), and HMGB1 using rabbit polyclonal (Abcam) antibody. Secondary antibodies conjugated to HRP for chemiluminescent detection were obtained from GE Healthcare.

### 2.7. RNA extraction and quantitative real-time PCR (qRT-PCR)

The mRNA expression of IL-8 and TNF-α were determined using quantitative Real-Time PCR.  $5 \times 10^5$  cells were plated in 6 mm plates. Cells were cultured for 24 h and then treated with different dust concentrations. Total RNA was extracted 12 and 24 h post dust exposure using a TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Total RNA was treated with RQ1 RNase-free DNase to eliminate contaminating genomic DNA. The total mRNA was converted into cDNA by reverse transcriptase PCR using the High-Capacity Reverse Transcription Kit (Applied Biosystems) according to the supplied protocol. The cDNA generated was used as a template in subsequent real-time PCR analyses. IL-8 and TNF-α primers are shown in Table S1. Transcript abundance was quantified by specific primers for IL-8 and TNF-α using a SYBR Green-based (Life Technologies) qPCR and normalized to Actin.

### 3. Results

#### 3.1. Dust leads to cell death with dying cells touching and engulfing more dust particles than surviving cells

We developed a live-cell imaging system to determine the effect of dust particles on individual cells over time. We imaged cells for 3 h before dust was added. We then exposed the cells to dust particles and imaged them for additional 48 h. We developed a semi-automated tracking method to track cells and quantified their growth and fate (Fig. 1C see Methods). We found that in response to dust concentration of  $25 \mu\text{g ml}^{-1}$ , 22% of the cells died, while the rest survived. We noticed that many cells directly touched dust particles and we, therefore, investigated whether local exposure to dust particles is linked with the onset of cell death. Fig. 1C shows an example of a cell at different times during our experimental measurements: before dust was added, a few hours after dust was added, showing three particles on the cell; and after cell division, showing dust particles on the two sister cells. We calculated the total time these particles were on the cells (referred here as 'Total Time Touching Dust Particles'; TTDP) and number of particles each cell interacted with. We calculated and compared the TTDP time for 50 cells that survived the dust treatment and 50 cells that died in response to dust. We found that dead cells showed longer TTDP compared to live cells (Fig. 1D). We noticed that the cells also physically reach out to engulf the particles (see examples in Movie S3). We therefore determined the relationship between cell fate and number of particles engulfed by cells over time (Fig. 1E). We found that most dust particles were engulfed by the cells in the first 2 h after the dust was added, and that dead cells show higher number of particles engulfed during these 2 h compared with surviving cells (Fig. 1E). Both populations continued to engulf particles throughout the entire 48 h with no significant difference between dead and live cells. Overall our results show that dead cells touch dust particles for longer periods and engulf a higher number of particles during the first 2 h post exposure to dust, compared to surviving cells, suggesting that local exposure to dust increases a cell's probability to die.

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.envres.2019.108891>.

#### 3.2. Exposure to dust reduces cell viability but leads to cell death in a non-monotonic way

We next tested the effect of different dust concentrations on cell growth and death. Specifically, we were interested in examining whether higher concentrations of dust will lead to more cell death. We first used a conventional approach for examining the effect of dust particles on cell viability by counting the number and size of human lung-cell colonies following exposure to different dust concentrations (Fig. 2A). We found reduction in both the number and sizes of colonies compared to a plate with no dust. As dust concentration increases the number and sizes of the colonies decrease (Fig. 2B–C), supporting previous studies showing that dust suppresses the growth and survival of human cells (Zeng et al., 2016; Yang et al., 2017).

We then used our live imaging system to measure the number of cells undergoing cell death under different dust concentrations. As we observed (Fig. 1) and was previously suggested (Rodríguez-Cotto et al., 2013; Chang et al., 2016), dust led to cell death. However, the relationship between dust concentrations and the percentage of dying cells was complex; at low dust concentrations (up to  $100 \mu\text{g ml}^{-1}$ ) increasing the concentration led to an increase in the percentage of dead cells (Fig. 2D). At higher doses ( $100 \mu\text{g ml}^{-1}$  -  $1000 \mu\text{g ml}^{-1}$ ) more dust led to a reduction in cell death with fewer cells dying in 500 and  $1000 \mu\text{g ml}^{-1}$  compared to 100 or  $250 \mu\text{g ml}^{-1}$ . These results show that indeed dust leads to cell death, however the relationship between dust concentration and the fraction of dead cells is non-monotonic.

To further study the dynamics of cell death, we quantified the time

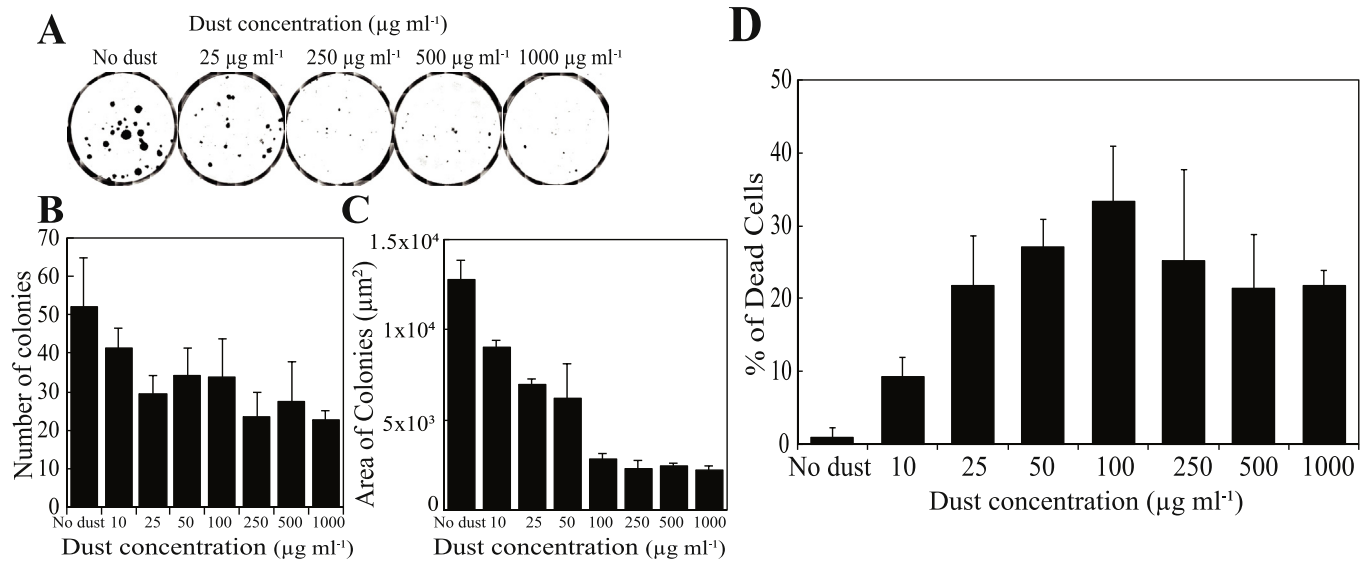
of death as a function of dust concentration. Specifically, we determined the percentage of cell death using 12 h intervals (Fig. S3). We found that the percentage of cell death at different dust concentrations varies between the different time intervals. Here again the relationship was non-monotonic. At the low ( $10 \mu\text{g ml}^{-1}$ ) and high ( $1000 \mu\text{g ml}^{-1}$ ) concentrations most cells died during the 36–48 h interval as opposed to the intermediate concentrations ( $25 \mu\text{g ml}^{-1}$  to  $500 \mu\text{g ml}^{-1}$ ) that showed most death by 36 h. Note that overall the percentage of cell death for  $100 \mu\text{g ml}^{-1}$  at 24 h was in agreement with previous studies (Veranth et al., 2007; Higashisaka et al., 2014; Chang et al., 2016) using different cell lines or dust types. Taken together our results suggest that cellular death may result from, and regulated by, multiple factors that operate differently and at different time scales under low and high dust doses.

#### 3.3. Dust reduces cell divisions and slows down the cell cycle

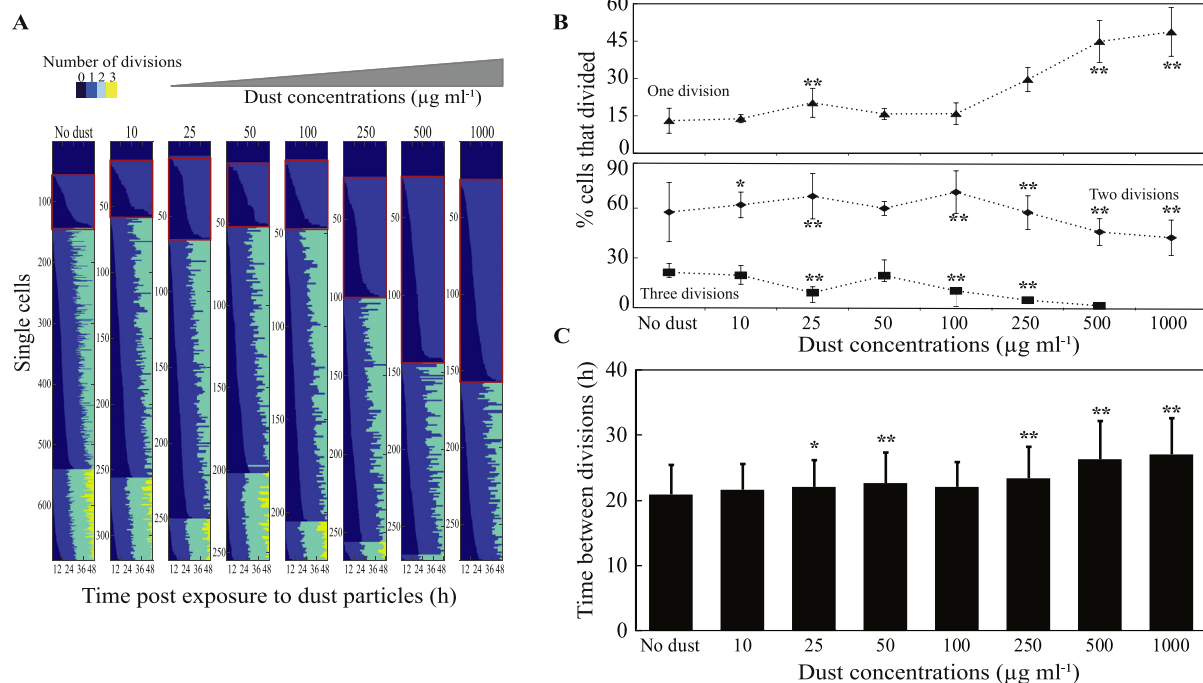
We and others have shown that dust reduces cell viability, with higher concentrations of dust leading to lower cell viability (Rodríguez-Cotto et al., 2013; Chang et al., 2016, and Fig. 2A–C). However, higher concentrations of dust did not lead to more cell death; in fact, under dust concentrations above  $100 \mu\text{g ml}^{-1}$  the fractions of dead cells were reduced (Fig. 2D). Cell death is only one mechanism affecting cell viability. The other is cell growth and proliferation. We therefore asked whether dust also leads to cell cycle arrest, which could explain the reduction of cell viability at high dust concentrations in the absence of increased cell death (Fig. 2D). We therefore used our live imaging system to directly quantify the effect of different dust concentrations on cell proliferation and the cell cycle. We quantified the number and timing of divisions that each surviving cell underwent during the 48 h following exposure to dust (Fig. 3A). Division profiles revealed large heterogeneity between single cells, ranging from cells that did not divide at all, cells that divided only once (single dividers Fig. 3A red squares), and cells that had multiple divisions (Fig. 3A). The proportions of cells in each category depended on the concentration of dust; on average, higher dust concentrations led to less divisions (Fig. 3B). To determine the effect of dust dose on cell cycle length we calculated the time between divisions for cells that divided at least 2 times under no-dust or different dust concentrations (Fig. 3C). We found that the time between cell divisions increased with dust concentrations. At higher dust concentrations it took the cells longer to divide compared to the untreated condition or lower concentrations (Fig. 3C and Table S2). Taken together our results show that dust affect cell growth by reducing the number of divisions and slowing down the cell cycle, with higher concentrations of dust leading to less divisions and longer time between divisions. Note that the effect on cell growth was more profound at dust concentrations of  $100 \mu\text{g ml}^{-1}$  and beyond (Fig. 3B), a concentration above which death was reduced (Fig. 2D). We therefore suggest that inhibition of the cell cycle can explain the non-monotonic death in response to different dust concentrations. Specifically, dust leads to both cell cycle arrest and cell death. At lower concentrations, mostly death is induced, while the inhibition of proliferation is minimal. At higher concentrations, proliferation is inhibited, which suppresses further death, and therefore leads to a lower fraction of dead cells.

#### 3.4. Cellular death in response to dust results from apoptosis and necrosis

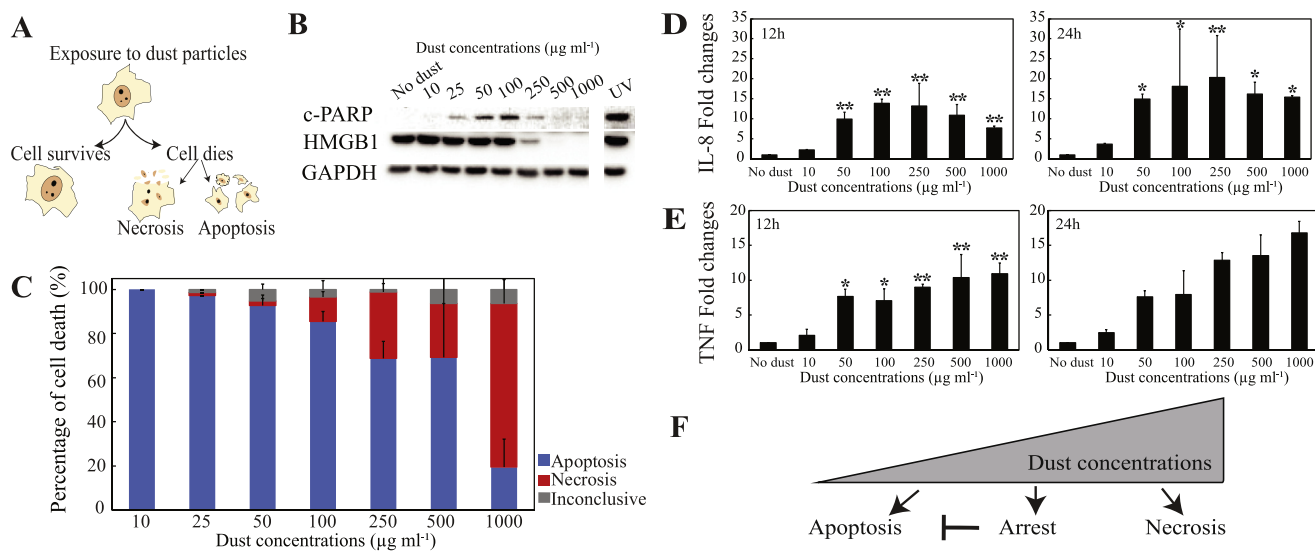
Previous studies suggested that necrosis is the main mechanism of cell death in response to dust particles (Schwarze et al., 2002; Hetland et al., 2004; Veranth et al., 2004; Rong et al., 2013). Our findings that cell death occurs during different time intervals under different dust concentrations (Fig. S3) suggested that different death mechanisms might operate under different concentrations (Fig. 4A). We used our live-imaging system and the morphology of single cells to identify cells that underwent necrosis. We indeed found cells that burst with membrane rupture, a hallmark of necrosis (see Movie S2A and Methods for



**Fig. 2.** Dust particles effect on cell viability, cell death behaves in a non-monotonic way when observed on a single cell basis, but a monotonic behavior observed at colonies basis. **(A)** Colonies imaged after exposure to different dust concentrations. **(B)** Reduction of average number of colonies with standard deviation values as a function of dust concentration. Each dust concentration represents three replicates. All dust cases were significantly different from no dust case (ANOVA,  $P < 0.001$ ). **(C)** Reduction of average size of colonies with standard deviation values as a function of dust concentration. Each dust concentration represents three replicates. All dust cases were significantly different from no dust case (ANOVA,  $P < 0.001$ ). **(D)** Percentage of cell death as a function of dust concentrations with standard deviation values, after 48 h. All dust cases were significantly different from no dust case (ANOVA,  $P < 0.001$ ). Data from a duplicate experiment showed a similar trend.



**Fig. 3.** High concentration of dust reduces cell divisions. **(A)** Division profiles obtained after tracking individual primary cells and annotating division in the course of 48 h after exposure to dust. Panels aggregate single cells exposed to a particular dust concentration. Each row represents the division profile of an individual cell over time. Colors change upon division events. Cells are grouped by their total number of division and ordered by the timing of their first division event. Red boxes highlight the single divider populations. **(B)** Divisions frequency of live cells as a function of dust concentration up to 48 h. Percentage of cells which divide only once (upper panel) and cells which divide twice and three times (lower panel) with standard deviations as a function of dust concentration. Asterisks indicate significantly different from the untreated condition (no dust) case based on ANOVA \* $P < 0.05$ ; \*\* $P < 0.001$ . Data from a duplicate experiment showed a similar trend. **(C)** Time between divisions (hours) as a function of dust concentration. Calculation of time between divisions, based on cells which divide more than two times. Asterisks indicate significantly different from the no dust (control) case based on ANOVA, \* $P < 0.05$ ; \*\* $P < 0.001$ . (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)



**Fig. 4.** Exposure to dust particles leads to different cellular death type and inflammatory responses. **(A)** Exposure to dust particles can lead to different cellular outcomes, including cell death via as apoptosis and necrosis. **(B)** Expression of C-PARP (apoptosis) and HMGB1 (necrosis) at high dust concentrations at 24 h post-dust addition at indicated concentrations. UV radiation represents positive controls for apoptosis. **(C)** Percentage of cell death type as a function of dust concentration with standard deviation. Type of cell death depends on dust concentration. As dust concentration increases the frequency of cells to die by necrosis (red) increases while that due to apoptosis (blue) decreases. **(D–E)** Dust particles effect the expression of IL-8 **(D)** and TNF- $\alpha$  **(E)**. Assessment the inflammatory responses induced in A549 cells by dust particles at different dust concentrations at two time points (12 and 24 h) using a qPCR. Fold change compared to the untreated case (no dust) for IL-8 and TNF- $\alpha$ . Values represent average and standard deviation of two biological repeats. Asterisks indicate significantly different from untreated condition based on ANOVA, \* $P < 0.05$ ; \*\* $P < 0.001$ . **(F)** Schematic summary of a proposal explaining our findings: apoptosis is the dominant mechanism of death under low dust concentrations. Higher dust concentrations induce cell cycle arrest, which inhibits apoptosis. Under high dust concentrations necrosis starts to dominate. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

more details). However, we also identified cells that showed separation to apoptotic bodies, suggesting apoptosis as an additional mechanism of cell death in response to dust (see Movie S2B). Western blot of the apoptotic marker c-PARP (Zhao et al., 2013) showed induction of c-PARP in response to dust, supporting apoptosis as an active mechanism of cell death following dust exposure (Fig. 4B). We next examined the cells' morphologies to determine which cells died from necrosis or apoptosis. We found that these percentages changed with different dust concentrations. At low dust concentrations apoptosis was the main death mechanism, but as dust concentration increases, the fraction of cells dying from apoptosis decreases and necrosis became the dominant death mechanism (Fig. 4C). These findings were supported by immunoblots for the apoptotic marker c-PARP and the necrosis marker HMGB1 under various dust concentrations (Fig. 4B). Note that HMGB1 is secreted from cells during necrosis (Raucci et al., 2007) and therefore reduction in cellular HMGB1 indicates the activation of necrosis. In agreement with our live imaging results, c-PARP showed a maximum level at  $100 \mu\text{g ml}^{-1}$  dust followed by a decrease at higher concentrations of dust (Fig. 4B). Cellular HMGB1 showed a minimal change in low dust concentrations and started to decrease at concentrations higher than  $100 \mu\text{g ml}^{-1}$  suggesting necrosis is induced under these high doses (Fig. 4B). The levels of pro-inflammatory cytokines, which are known to be upregulated in response to dust (Veranth et al., 2004; Rong et al., 2013), supported these findings. The levels of IL-8, which is known to be induced by both apoptosis (Cullen et al., 2013) and necrosis (Namba et al., 2017) showed a non-monotonic induction in the first 12 h, and a more continuous increase at 24 h post dust exposure. The levels of TNF- $\alpha$ , which is secreted in necrosis (Hitomi et al., 2008; Nikolettou et al., 2013) showed little increase in the first 12 h and a gradual dose-dependent increase at 24 h (Fig. 4D–E). These results suggest that death in response to dust results from a combination of apoptosis and necrosis. Apoptosis is the dominant mechanism of death

under low dust concentrations. As dust concentration increases, cell cycle arrest is induced and inhibits apoptosis. At high concentrations of dust, necrosis also starts operating and dominate the mechanism of death (Fig. 4F).

#### 4. Discussion

Exposure to dust particles is known to affect cells' viability. Previous studies investigated this by focusing on cell populations, and quantifying thousands of cells at single fixed timepoints at usually 24 h (Yamada et al., 2012; Chang et al., 2016; Pardo et al., 2017) or 48 h (Deng et al., 2007; Zeng et al., 2016) post dust exposure. In this work, we used live imaging to follow individual cells over time in response to a wide range of dust concentrations. This technique allowed us to study the timing and mechanism of cell death as well as the effect of dust on the cell cycle length and proliferation. One observation from our work is that there were minimal dead cells in the first 12 h post dust treatment, even under high concentrations of dust ( $25\text{--}1000 \mu\text{g ml}^{-1}$ ). This time lag can explain the finding of several epidemiological studies showing an increase in emergency room visits a day or two after dust storms (Middleton et al., 2008; Vodonos et al., 2015; Crooks et al., 2016).

Phagocytosis is an important mechanism for clearance of dust particles from the lungs. Previous studies observed the capability of A549 cells to engulf particles (Stearns et al., 2001; Jordan et al., 2009; Wang et al., 2009). Clusters of particles were observed on the surface of the cells (Jordan et al., 2009), around the nuclear region, as well as near the basal end of the cell (Stearns et al., 2001). Here we quantified engulfment of dust particles in real time and linked it with cell death. Our results showed that most of the particle engulfment occurs during the first 2 h of exposure to dust, in agreement with Geiser et al. (1990) that showed rapid phagocytosis. Our work also showed that engulfment of

dust particles continues throughout the entire duration of our experimental measurement (48 h).

Our work revealed a complex relationship between dust concentrations and cell death. Previous studies, looking at cell viability, suggested a monotonic relationship (Veranth et al., 2004, 2006; Yamada et al., 2012; Rodríguez-Cotto et al., 2013; Chang et al., 2016). Here we looked specifically at cell death and found a non-monotonic relationship between dust concentration and the fraction of dead cells. Based on our measurements on the cell cycle and growth, we suggest that the decrease in cell death at high dust concentrations is explained by the induction of cell cycle arrest, which suppresses cell death (Pucci et al., 2000).

Using our live single cell imaging system, we found that cell death was a mixture of necrosis and apoptosis. Apoptosis was the dominant mechanism of death at low dust concentrations and decreases as the dust concentration increases, while necrosis increases as the dust concentration increases. While apoptotic cells are generally believed to be immunologically silent and do not provoke inflammation (Krysko et al., 2008), necrotic cells release their cellular contents into the surrounding extracellular space and trigger an inflammatory response (Duprez et al., 2009). The mRNA levels of TNF- $\alpha$ , a necrotic marker, mimicked the pattern of necrosis with a gradual increase as the dust concentrations increased, as has been observed before (Hitomi et al., 2008; Nikolettou et al., 2013; Rong et al., 2013). In contrast, IL-8 mRNA levels increased as a function of dust concentration up to 100–250  $\mu\text{g ml}^{-1}$  and then decreased at higher concentrations, similar to the overall death trend. This non-monotonic behavior of IL-8 has been observed for other particles types and concentrations and for different cell lines (Hetland et al., 2004; Veranth et al., 2006). IL-8 is affected by both necrosis (Namba et al., 2017) and apoptosis (Cullen et al., 2013); therefore, its non-monotonic behavior is a mixture of both mechanisms and its levels mimic the fraction of cell death observed under various dust concentrations.

During the last decade, large attention has been given to mineral dust particles from desert dust storms. Although epidemiological studies from affected areas suggest the potential health effects of desert dust, the evidence remains unclear (Tobias et al., 2019) since previous reports had inconsistent results across studies and different geographical regions (Hashizume et al., 2010; Karanasiou et al., 2012; de Longueville et al., 2013; Zhang et al., 2016). In addition, the biological mechanisms responsible for health implication caused by the penetration of dust particles are still unclear (Deng et al., 2007; Yang et al., 2017). With further usage of single cell approaches we believe that we will be able to improve our understanding of the health-related consequences of exposure to particles from desert dust storms.

In this work we studied a specific dust particle type (Montmorillonite). Dust particles of different types and sizes may have different effects on cells including their effect on proliferation, mechanism of death and proinflammatory cytokines. In addition, we focused here on the lung cancer cell line A549. While this line has been widely used in many pulmonary studies, and was shown as a suitable substitute to primary or stem cells of the alveolar epithelium (Fernando et al., 2019) and to mimic major functionalities of primary alveolar type II cells (Cooper et al., 2016), it is still unclear whether other cancerous or healthy cell lines will show similar or different behaviors, which will be an interesting follow up of this work. Our study raises many additional questions; what determines if a cell will die from apoptosis or necrosis? Why do human cells engulf dust particles and what molecular mechanisms link dust phagocytosis with cell death? Using live single cell imaging approaches together with reporters of specific signaling pathways will be essential for starting to address these questions in order to develop a better understanding on the relationship between dust particles and cellular outcomes.

## 5. Conclusions

This work provides a quantitative look at the effect of dust storm particles (Montmorillonite) on human epithelial cells (A549) and their interactions at a single cell level. Live-cell imaging allowed us to monitor cell number and timing of divisions, as well as time and mechanism of cell death in response to wide concentrations of dust. Our findings revealed that dust concentration has an effect on cellular outcomes with a large cell-to-cell variability; some cells survive while others die, even in response to the same dust concentration. We also concluded that local exposure to dust increases a cell's probability to die, since cells that died interacted with dust particles for longer times, and engulfed more dust particles, compared to surviving cells. In general, we concluded that higher dust concentrations inhibit cell proliferation and reduce viability; however, the effect on cell death is non-monotonic. Using cellular morphological, immunoblots and profiling of proinflammatory cytokines, we determined that apoptosis was dominant at low dust concentrations, while necrosis was dominant at higher dust concentrations. Our study is in agreement with previous work showing that dust reduces cell viability, while providing a quantitative, high resolution picture, both in time and single cell level, to better understand which mechanism of reduced viability (e.g. cell cycle arrest, apoptosis, necrosis) operates under a specific dust concentration.

## Acknowledge

We thank members of the Lahav and Ardon-Dryer labs for useful comments and discussions of the work. This work was funded by grants NIH GM083303 and GM116864. J.R. received support from CONACyT/Fundacion Mexico en Harvard (404476), and Harvard Graduate Merit Fellowship.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.envres.2019.108891>.

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