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## Effects of Eyjafjallajökull Volcanic Ash on Innate Immune System Responses and Bacterial Growth *in Vitro*

Martha M. Monick<sup>1</sup>, Jonas Baltrusaitis<sup>2</sup>, Linda S. Powers<sup>1</sup>, Jennifer A. Borcharding<sup>1</sup>, Juan C. Caraballo<sup>1</sup>, Imali Mudunkotuwa<sup>2</sup>, David W. Peate<sup>3</sup>, Katherine Walters<sup>4</sup>, Jay M. Thompson<sup>5</sup>, Vicki H. Grassian<sup>2</sup>, Gunnar Gudmundsson<sup>6</sup>, and Alejandro P. Comellas<sup>1</sup>

<sup>1</sup>Department of Medicine, Carver College of Medicine, University of Iowa, Iowa City, Iowa, USA

<sup>2</sup>Department of Chemistry, University of Iowa, Iowa City, Iowa, USA

<sup>3</sup>Department of Geoscience, University of Iowa, Iowa City, Iowa, USA

<sup>4</sup>Central Microscopy Research Facility, University of Iowa, Iowa City, Iowa, USA

<sup>5</sup>ARC Centre of Excellence in Ore Deposits, University of Tasmania, Hobart, Tasmania, Australia

<sup>6</sup>University of Iceland, Reykjavik, Iceland

Corresponding author: Martha M. Monick, PhD, Division of Pulmonary, Critical Care, and Occupational Medicine, Room 100, EMRB, University of Iowa, Iowa City, IA 52242, Phone: (319) 335-7590, Fax: (319) 335-6530, Email: [martha-monick@uiowa.edu](mailto:martha-monick@uiowa.edu)

**Short title:** Volcanic ash impairs innate immunity

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**Abbreviations:** ASL, airway surface liquid; AMPs, antimicrobial peptides; COPD, chronic obstructive pulmonary disease; Gt, Transepithelial electrical conductance; ICP-MS, inductively coupled plasma mass spectrometer; PA01, *Pseudomonas aeruginosa*; SEM, Scanning electron microscopy; XPS, X-ray photoelectron spectroscopy

## Abstract

**Background:** On March 20, 2010, the Icelandic volcano, Eyjafjallajökull, erupted for the first time in 190 years. Despite many epidemiological reports showing volcanic ash effects on the respiratory system, there is limited data evaluating cellular mechanisms involved in the response to ash. Epidemiological work shows an increase in respiratory infections of subjects and populations exposed to volcanic eruptions.

**Methods:** Volcanic ash was physicochemically characterized, demonstrating various sizes and presence of several transition metals, including iron. The effect of Eyjafjallajökull ash on primary alveolar and airway epithelial cells (20 to 100  $\mu\text{g}/\text{cm}^2$ ), human alveolar macrophages (5 to 20  $\mu\text{g}/\text{cm}^2$ ) and on *Pseudomonas aeruginosa* (PAO1) growth (2  $\mu\text{g}/10^4$  bacteria) were tested.

**Results:** Volcanic ash had minimal effect on alveolar and airway epithelial cell integrity. In alveolar macrophages, volcanic ash disrupted pathogen killing and inflammatory responses. Volcanic ash, in *in vitro* bacterial growth models, increased bacterial replication and decreased bacterial killing by anti-microbial peptides.

**Conclusions:** These results provide potential biological plausibility for epidemiological data that associates exposure to air pollution with the development of respiratory infections. These data support the conclusion that volcanic ash exposure, while not seriously compromising lung cell function, may impair innate immunity responses in exposed individuals.

## Introduction

On March 20, 2010, the Iceland volcano, Eyjafjallajökull, erupted for the first time in 190 years from a vent on its flank (Swindles et al. 2011). A second and larger eruption occurred on April 14<sup>th</sup> from the summit, continuing until late May 2011 (Sigmundsson et al. 2010). An interaction between ash and magma led to large volumes of finely comminuted ash entering the atmosphere (Gudmundsson 2011). The eruption plume was approximately six miles high, leading to significant ash dispersal to both Iceland and parts of Europe (Gislason et al. 2011). The average ash concentration in the cloud that reached Europe has been calculated at 10-35 mg/m<sup>3</sup>, with peak ambient air concentrations of particulate matter as high as 13 mg/m<sup>3</sup>. Even after the eruption ceased, ambient air concentrations of ash were close to 1 mg/m<sup>3</sup> (Thorsteinsson 2012). Approximately 35% of particles was under 30 micrometer in size during the April 14-16<sup>th</sup> initial explosive phase (Gudmundsson et al. 2012). Ash collected immediately after the eruption was found to have up to 25% respirable particles (less than 10 microns) (Gislason et al. 2011). In addition to particles, the gaseous component included sulfur dioxide and other species that may harm human health in proximity to the eruption (Schmidt et al. 2011).

While volcanic eruptions are a fairly rare event, there is significant data supporting the adverse health effects of respirable particles. A report in 1973 linked air pollution and premature mortality (Lave and Seskin 1972). This association has been confirmed in subsequent studies (Dockery et al. 1993; Elliott et al. 2007; Samet et al. 2000; Spix et al. 1993). Volcanic eruptions, because of the significant particle burden in the atmosphere, have adverse effects on human health, including bronchitis, asthma exacerbations and hospital admissions due to respiratory symptoms (Baxter et al. 1981; Baxter 1983).

During the 1980 Mount St. Helens eruption, Washington State, US, several people died from asphyxia by volcanic ash and thermal burn with airway injury. Subsequent reports looked at the sub-acute and chronic effects of Mount St. Helens eruption. Asthma exacerbations, upper respiratory infections, otitis, and bronchitis were documented (Bernstein et al. 1986). Some Mount St Helens studies found only limited risk of lung infections (Martin et al. 1986). In the Icelandic eruption, Carlsen et al reported that Icelanders exposed to Eyjafjallajökull volcanic ash had increase prevalence of respiratory symptoms, specifically asthma and chronic bronchitis, compared to a control population in the north of Iceland (Carlsen et al. 2012). While, other reports have also shown that exposure to volcanic ash increases the risk of developing respiratory infections (Convit et al. 2006; Gudmundsson 2011; Naumova et al. 2007), there is limited data evaluating the cellular mechanisms involved in the increase risk of lung infections after exposure to volcanic ash. Since airway infections are the product of impaired innate immune mechanisms, we hypothesized that volcanic ash will impair innate immune mechanisms, specifically macrophage and antimicrobial peptides function.

## **Methods**

**Volcanic ash collection and characterization.** Three batches of ash were collected shortly following the Eyjafjallajökull eruption in 2010. Ash A was collected May 5 about 38 km from the source at Vik village by rescue team member Brydis Hardottir and sent to the Institute for fluorine and grain size measurement. Ash B was collected about 58 km from source as ash fell on morning of April 15 by Sigurdur Gislason, geologist at the Institute of Earth Sciences, University of Iceland, Reykjavik, Iceland. Ash C was collected about 64 km from the source by Porarinn Eggertsson, farmer at Hraungerdi, and sent to the Institute for fluorine and grain size

measurement. Except for experiments using all three batches of ash, all other experiments (including the sieved experiments) were done using batch B. Characterization of volcanic ash was determined by Scanning Electron Microscopy (SEM), X-ray Photoelectron Spectroscopy (XPS), and Inductively Coupled Plasma Mass Spectrometer (ICP-MS) (see Supplemental Material, Figure S1, Tables S1 and S2).

**ICP-MS.** Samples were analyzed for elemental concentrations using a Thermo X-Series II quadrupole inductively coupled plasma mass spectrometer (ICP-MS) instrument, following the methods described in Peate et al (Peate et al. 2010). Ash samples were dissolved using a standard HF-HNO<sub>3</sub> acid digestion method.

Leaching experiments were carried out in 0.001M ultrapure HNO<sub>3</sub>, using an ash (g) to acid (ml) ratio of 1:25 (0.1 g ash to 2.5 mL water/acid), following the recommendations of Witham et al. (Witham et al. 2005). The ash-leach mixtures were agitated by shaking for 90 min in a sealed polyethylene bottle using an ultrasonic bath. Leachates were filtered through a 0.45µm cellulose acetate Millipore filter prior to analysis. Data were corrected for blanks and instrumental drift and calibrated relative to a series of multi-element solutions (1 ppb, 10 ppb, 50 ppb) gravimetrically-prepared using ultra-pure Milli-Q water from a 1,000 ppm stock solution (Inorganic Ventures). Accuracy was assessed by comparing analyses of two natural water standard reference materials (NIST1640a and SLRS-5) analyzed as unknown samples with their certified values. Final leaching data are presented as elemental concentrations in mg per kg of ash. The complete ICP-MS data set, including standards, is provided in Supplemental Material, Tables S1 and S2.

**X-ray photoelectron spectroscopy (XPS).** A custom-designed Kratos Axis Ultra X-ray photoelectron spectroscopy (XPS) system (Kratos Analytical, Chestnut Ridge, NY) was used (Baltrusaitis et al. 2007). The surface analysis chamber is equipped with an aluminium  $K\alpha$  source using a 500 mm Rowland circle silicon single crystal monochromator giving monochromatic radiation at 1486.6 eV. The X-ray gun was operated using a 15 mA emission current at an accelerating voltage of 15 kV. High resolution spectra were acquired in the region of interest using the following experimental parameters: 20 to 40 eV energy window; pass energy of 20 eV; step size of 0.1 eV and dwell time of 1000 ms and X-ray spot size of 700x300  $\mu\text{m}$ . Low energy electrons were used for charge compensation to neutralize the sample. One sweep was used to acquire region spectra. The absolute energy scale was calibrated to the Cu  $2p_{2/3}$  peak binding energy of 932.6 eV using an etched copper plate. All spectra were charge referenced to C 1s at 285.0 eV. Samples containing particles were mounted on indium foil. CasaXPS software was used to process the XPS data. A Shirley-type background was subtracted from each spectrum to account for inelastically scattered electrons that contribute to the broad background. Transmission corrected relative sensitivity factor (RSF) values from the Kratos library (<http://www.casaxps.com/kratos/>) were used for elemental quantification, as implemented into CasaXPS.

**Particle preparation for in vitro experiments.** Sieved ash (20  $\mu\text{M}$ ) from the Eyjafjallajökull eruption was suspended in media identical to the cell culture media with the addition of dipalmitoylphosphatidylcholine (DPPC) (10  $\mu\text{g/ml}$ ). Particle suspensions were sonicated for 20 seconds immediately before adding to cell cultures. In some cases other particles were also used for cell exposures (aluminum oxide ( $\text{Al}_2\text{O}_3$ )). They were prepared in an identical manner.

**Cell Models.** Cells used in these experiments included primary human and rat alveolar macrophages, primary rat alveolar epithelial cells and primary human airway epithelial cells. Human alveolar macrophages were obtained from recruited healthy nonsmoking subjects (age 20-40, equally divided between males and females). Bronchoalveolar lavage was performed by instilling 20 ml of normal saline into a tertiary bronchus up to five times in three different lung segments. Slides were microscopically examined to ensure that greater than 95% of the cells were macrophages (Monick et al. 2006; Monick et al. 2008; Monick et al. 2010). All procedures and protocols described in this communication have complied with all applicable requirements of the U.S.A. and/or international regulations (including IRB approval). All human participants gave written informed consent prior to the study. Rat alveolar macrophages were isolated from pathogen-free male Sprague-Dawley rats weighing 200-225g. Animals were purchased from Harlan Laboratories (Madison, WI). All animals were housed under standard conditions (50% humidity and 12h-dark- 12h-light cycle) at the University of Iowa animal facility. Fresh water and food was available ad lib. The alveolar macrophages were isolated and purified by differential adherence to immunoglobulin G-coated dishes. BAL fluid was spun and cell pellet was resuspended in antibiotic free DMEM 10% FBS. Cells were plated and allowed to adhere for one hour before conducting experiments. Primary rat alveolar type II epithelial cells were isolated from male Sprague Dawley rats as previously described (Dobbs et al. 1986). Briefly, the lungs were perfused via the pulmonary artery, lavaged and digested with elastase (30 U/ml; Worthington Biochemical) for 20min at 37°C. The ATII cells were purified by differential adherence to immunoglobulin G-coated dishes. Counting and viability of the ATII cells were assessed by exclusion of trypan blue stain. The animals were treated humanely and with regard

for alleviation of suffering. Human airway epithelial cells were obtained from the University of Iowa cell culture core and were seeded as previously described (Karp et al. 2002).

**Culture conditions.** For macrophages, cells were cultured at 1 million/ml in standard tissue culture flasks (60 mM and 100 mM, RPMI 1640 with gentamycin). Epithelial cells were plated with Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 2mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 2.5 µg/ml of amphotericin. All airway epithelial cells were placed on filters with the purpose of developing cell polarity. Experiments were performed three days after isolation.

**Volcanic ash particle exposure concentrations.** All cells were exposed to particles as a function of surface area ( $\mu\text{g}/\text{cm}^2$ ), while bacteria was standardized as µg of ash particles per number of bacteria ( $\mu\text{g}/10^4$  PAO1).

**Scanning electron microscopy (SEM).** Isolated rat or human macrophages were exposed to volcanic ash for 2 hours and fixed overnight with 2.5% glutaraldehyde (Sabatini et al. 1963) in 0.1 M cacodylate buffer. After standard processing protocols were completed, imaging was performed with a Hitachi S-4800 field emission scanning electron microscope (Hitachi High Technologies America, Inc. Schaumburg, IL).

**Western analysis.** Whole cell protein was obtained as previously described (Philibert et al. 2012). Following protein purification, Western analysis was performed following protocols as previously described (Reisetter et al. 2011). The following antibodies used were purchased from Cell Signaling Technology Inc. (Danvers, MA): LC3 (2775), Ubiquitin (3936), phospho ERK (9101), phospho p38 (9215). Phospho JNK (559309) antibody was purchased from EMD

Millipore (Billerica, MA) and beta actin (ab8226) was purchased from Abcam (Cambridge, MA).

**Cell death measurements.** Cell death measurements were done with propidium iodide 10 $\mu$ g/ml in PBS or trypan blue (Reisetter et al. 2011). Pictures were analyzed with Image J software (an open source software developed by Wayne Rasband, Research Services Branch, National Institute of Mental Health, Bethesda, Maryland, USA).

**Transepithelial electrical conductance (Gt) measurement.** Electrical resistance was measured with the Millicell Electrical Resistance System (ERS) (Millipore Corporation, Bedford, MA) and conductance (Gt) was calculated as its reciprocal.

**Bacterial growth assays.** *Pseudomonas aeruginosa* (PAO1) grown overnight in M9 media (1x M9 Salts, 2.2 mM glucose, 0.002 M Magnesium Sulfate (MgSO<sub>4</sub>), 0.001 M Calcium Chloride (CaCl<sub>2</sub>) and 25mM sodium succinate) was exposed to FeCl<sub>3</sub> (10 $\mu$ g/mL), a soluble source of iron, Al<sub>2</sub>O<sub>3</sub> (10 $\mu$ g/mL) to control for particle effects and volcanic ash (10 $\mu$ g/mL). Growth was recorded over nine hours at 37°C by measuring OD<sub>600</sub> and adjusting for particle absorbance effects. Data were compared for all parameters of the growth curve using the extra sum of squares F-test to detect differences throughout the entire curve.

**Bacterial killing assay.** Isolated rat or human macrophages were exposed to 25  $\mu$ g/mL volcanic ash for two hours and primed with 10ng/mL LPS for one hour. Cells were exposed to PAO1 (2.5e6 CFU). At 20 minutes and 110 minutes, cells were washed with 4 x Hanks and harvested. Cells were then lysed in ice cold ddH<sub>2</sub>O. Samples were plated and CFUs determined. Effect of bacterial phagocytosis was determined at 20 minutes and killing was determined at 110 minutes, by comparing PAO1 CFUs between volcanic ash and control.

**Antimicrobial peptide activity.** PAO1 was grown overnight in M9 media, subcultured and diluted to OD<sub>600</sub>=0.45. The culture was then diluted 1/1000 and 10µl added to start experiment. Sodium phosphate buffer at pH 7.8 was used and a cocktail of antimicrobial peptides (600µg/mL Lysozyme, 200µg/mL Lactoferrin and 100ng/mL β-Defensins 1&2) equaling 400µl were added to a 96 deep well plate. 10µg/mL CFA was added with AMPs and PAO1. Mixture was incubated for one hour at 37°C and 300 rpm. 25% Luria Broth (LB) media was added to mixture and grown overnight. OD<sub>600</sub> was measured to determine level of antimicrobial peptide activity. CFUs were determined by conducting the above experiment, serially diluting and plating cultures on LB agar plates at beginning and endpoints to determine exact colony count.

**Statistical analysis.** Unpaired Student t-test and one-way ANOVA were used to determine significance between experimental groups. Data are presented as means ± SEM. The program used for data analysis was GraphPad Prism 5.00 (San Diego, CA).

## Results

**Characterization of volcanic ash.** SEM image of volcanic ash particles, sieved through a <20 µm sieve were obtained: see Supplemental Material, Figure S1. There is a distribution of particle sizes that can be seen with two distinct fractions, 20 µm and less than ~2 µm. To obtain information about the surface elemental composition, XPS analysis was performed and the data can be seen in Supplemental Material, Table S1. It can be seen that volcanic fly ash is comprised of aluminosilicates with detectable concentration of biologically relevant metals such as iron. From Figure S1B, it can be seen that metals such as iron and titanium are present in localized areas. ICP-MS element concentrations (in mg per kg ash) for the unsieved ash sample and the < 20µm sieved fraction were done: see Supplemental Material, Table S2. There is excellent

agreement between ICP-MS compositional analyses presented here of three separate unsieved ash samples and the average of published data (Borisova et al. 2012; Sigmarsson et al. 2011). Leaching experiments were also performed to better understand the propensity of the Icelandic volcanic ash towards dissolution in aqueous environments. For iron, leaching the bulk ash in water released about 2 mg Fe per kg ash, leaching the bulk ash in weak acid released about 33 mg Fe per kg ash, while leaching the < 20  $\mu\text{m}$  fraction released 900 mg Fe per kg ash (see Supplemental Material, Table S2).

**Interaction of volcanic ash with human tracheobronchial epithelium and human alveolar macrophages.** To examine the effect of ash from the Eyjafjallajökull eruption on the respiratory system, primary rat alveolar epithelial cells and primary human alveolar macrophages were exposed to ash in culture. Figure 1A shows rat alveolar epithelial cells and ash particles sitting down on the epithelial surface of alveolar epithelial cells. For alveolar macrophages, Figure 1B demonstrates ash particles adherent to the surface. Figure 1C shows ash particles being internalized by alveolar macrophages and in vesicles within the cell. Figure 1D shows STEM/EDS elemental analysis of particles inside alveolar macrophages showing they are rich in elements, typical to those of volcanic ash.

**Functional studies of volcanic ash on primary lung cells.** For human alveolar macrophages, we tested the effect of ash on homeostatic mechanisms (autophagic vesicles and clearance of ubiquitin tagged proteins). In addition, we tested the result of concordant ash and endotoxin (LPS) exposures and determined if the presence of ash altered bacterial killing by human alveolar macrophages. Autophagy is an important cellular homeostatic mechanism that clears particulates, protein aggregates, old and damaged mitochondria and cytosolic bacteria (Gutierrez et al. 2004; Monick et al. 2010). ATG8 (LC3) is the most widely monitored autophagy marker.

We tested alveolar macrophages exposed to three batches of ash ( $20 \mu\text{g}/\text{cm}^2$  of A, B, C, see methods) using Western analysis. Figure 2A demonstrates increased levels of LC3-II with all the ash exposures. This suggests that ash is either increasing generation of autophagosomes or blocking progression of autophagosomes to fusion with lysosomes. To test which is occurring, we analyzed total ubiquitin levels by Western analysis. Figure 2A demonstrates an accumulation of ubiquitin conjugated proteins with ash exposure. These data suggests that ash exposure interferes with the process of autophagy in human alveolar macrophages.

To test the effect of ash exposure on inflammatory pathways, we measured activation of MAP kinase pathways and cytokine production after LPS exposure. Figure 2B shows LPS-induced activation of ERK, p38 and JNK (as shown by an increase in phosphorylation of the activated tyrosine and threonine sites) that was inhibited by ash exposure. All three pathways are required for optimal cytokine responses in these cells (Carter et al. 1999a; Carter et al. 1999b; Monick et al. 1999). Ash exposure led to a decrease in activation of JNK and ERK by LPS, while having no effect on p38 activity. Because LPS-induced ERK and JNK activity was inhibited, we tested the effect of ash exposure on TNF $\alpha$  mRNA expression after LPS. Figure 2C shows that ash decreases the production of TNF $\alpha$  mRNA by LPS when alveolar macrophages have been exposed to ash.

To determine whether Eyjafjallajökull volcanic ash impairs the ability of macrophages to kill bacteria, we determined PAO1 killing by alveolar macrophages treated with and without volcanic ash. We first tested phagocytosis of bacteria after a pre-incubation with ash and found no difference in bacterial uptake between control macrophages and ash exposed macrophages (Figure 2D). For bacterial killing, Figure 2E shows a trend towards significantly less bacteria in the volcanic ash group than in the control ( $p=0.07$ ). Interestingly, this was a concentration

dependent effect, since  $5\mu\text{g}/\text{cm}^2$  impaired macrophage ability to kill bacteria, while at a concentration of  $2\mu\text{g}/\text{cm}^2$  there was no difference with control condition (data not shown).

For lung epithelial cells, we tested whether volcanic ash would induce epithelial barrier disruption or cell death of alveolar or airway epithelial cells. Figure 3A shows that volcanic ash does not induce cell death in either alveolar or airway epithelial cells. Several authors have reported that particulate matter induces disruption of the epithelial barrier integrity (Caraballo et al. 2011; Petecchia et al. 2009; Slebos et al. 2007; Soberanes et al. 2009; Upadhyay et al. 2003). Both alveolar and airway epithelial cell barrier integrity was preserved in the presence of volcanic ash (Figure 3B). Therefore, these results demonstrate that Eyjafjallajökull volcanic ash does not induce alveolar epithelial injury, even at high concentrations. This is different than studies on other particulates, where in our lab both ambient air particles and diesel exhaust particles were found to increase transepithelial conductance (Caraballo et al. 2011; Caraballo et al. 2012)

**The effect of volcanic ash on bacterial growth and airway killing capacity.** The Eyjafjallajökull volcanic ash is rich in iron ( $\sim 75,000\text{ mg/kg}$  ash): see Supplemental Material, Table S2. On a global scale, it has been demonstrated that volcanic ash can release readily soluble iron into the environment (Olgun et al. 2011). To test if volcanic ash increased bacterial growth,  $3\mu\text{g}/10^4$  bacteria of sieved volcanic ash particles were added to three hour sub-cultured PAO1 cultures and growth was observed.  $\text{FeCl}_3$  ( $25\mu\text{M}$ ), a soluble iron source, was used solely as positive control. As shown in Figure 4A, Eyjafjallajökull volcanic ash particles increased bacterial growth compared to control and an iron deficient particle ( $\text{Al}_2\text{O}_3$ ) ( $p < 0.0001$ ). This result suggests that volcanic ash can be a bioavailable source of iron for PAO1 growth.

In the lungs, antimicrobial peptides are located in airway surface liquid (ASL). ASL is comprised primarily of lactoferrin, lysozyme, and  $\beta$ -Defensins 1&2. Lysozyme degrades the bacterial cell wall,  $\beta$ -defensins have broad antibacterial activity and lactoferrin sequesters iron and inhibits microbial respiration (Wiesner and Vilcinskas 2010). To test whether volcanic ash inhibits antimicrobial peptide activity, we combined physiologically relevant concentrations of antimicrobial peptides with volcanic ash ( $3\mu\text{g}/10^4$  PAO1) or  $\text{FeCl}_3$  and observed the effect on PAO1 growth (see schematic representation Figure 4B). As shown in Figure 4C, in the presence of antimicrobial peptides, PAO1 did not grow, indicating complete bacterial killing by the antimicrobial peptide cocktail. The inhibitory effect of antimicrobial peptides on PAO1 growth was compromised when volcanic ash was added to the system (Figure 4C, blue bars,  $p \leq 0.0001$ ). Thus, volcanic ash inhibits antimicrobial peptide activity.

## Discussion

Close to 10% of the world population lives within 100 km of historically active volcanos. Of all the potential hazards of an eruption, ashfall can affect people the most because of the wide areas covered by fallout and the length of the exposure. Although eruptions are often short-lived, ashfall deposits can remain in the local environment for years to decades, being remobilized by human activity or simply re-suspended by wind (Hansell et al. 2006).

Several groups have reported that populations exposed to volcanic air pollution have increased prevalence and incidence of upper and lower tract respiratory infections (Amaral and Rodrigues 2007; Longo and Yang 2008; Naumova et al. 2007). The Mount St. Helens eruption was one of the most studied eruptions in terms of potential health effects of volcanic activity. During the next two weeks, there was a significant increase in emergency room visits at this location. The

major reasons for emergency room visits were upper respiratory infections (URIs) and otitis (approximately a 600% increase) (Bernstein et al. 1986). Grose et al (1985) reported that intratracheal instillation in mice of both fine and coarse Mount St. Helens volcanic ash caused small but a significant increase in susceptibility to streptococcal infections when the ash was instilled 24 hours prior to bacterial challenge (Grose et al. 1985). A recent study by Carlsen et al reported that Icelanders exposed to Eyjafjallajökull volcanic ash had increase prevalence of respiratory symptoms (Carlsen et al. 2012). The effects of volcanic ash on bacteria growth and innate immunity were not definitively established.

Immediately after the Icelandic eruption, peak particulate concentrations were as high as 13 mg/m<sup>3</sup> and even after the eruption ceased, they were measurements close to 1 mg/m<sup>3</sup> (Thorsteinsson 2012). Taking these two concentrations, and assuming a minute ventilation of 6 l/min (approx 8.6 m<sup>3</sup> over 24 hours) for a healthy adult at rest, the total dose inhaled over 24 hours would be 111.8 mg for the peak particulate concentration (13 mg/m<sup>3</sup>) and 8.6 mg for the 1 mg/m<sup>3</sup> concentrations. However, since it is difficult to predict whether an exposed individual may have used nasal, oral-nasal, etc. breathing patterns, and to predict the size distribution of particulates inhaled, it is safe to assume that only 50% is deposited in the lung. Assuming that human airway surface area is 4,430 cm<sup>2</sup>, in the first day a healthy subject will accumulate close to 126 µg/cm<sup>2</sup> particulates during the peak concentration, and 1 µg/cm<sup>2</sup> of particulates when exposed to 1 mg/m<sup>3</sup>. The airway epithelial cells experiments used concentrations of 10, 50 and 100 µg/cm<sup>2</sup>. Therefore these concentrations are within the range of what subjects could have been exposed in the immediate days after the eruption. Ash collected immediately after the eruption was found to have up to 25% respirable particles (less than 10 microns) (Gislason et al.

2011), which is the relevant particle portion that reaches the lung. Most of our experiments were performed with volcanic ash closer to the respirable particle fraction, (<20 micron).

Macrophage function is impaired by exposure to volcanic ash, specifically bactericidal activity and not phagocytosis. It is likely that the ash overwhelms the capacity of macrophages to kill bacteria. At lower concentrations, ash inhibited antimicrobial peptide activity, thus impairing bactericidal and bacteriostatic activity of the airway. Since metal content is abundant in volcanic ash as well as fly ash, our findings have implications to work published on the effect of fly ash and innate immunity. For example, Roberts et al examined the effect of soluble metal fraction of residual oil fly ash on immunity to *Listeria Monocytogenes*. They found a link between nickel from fly ash and reduced lung immunity in a rat model (Roberts et al. 2009). Also, Klein-Patel et al showed that residual oil fly ash inhibits beta defensin gene expression in bovine and human lung epithelial lines (Klein-Patel et al. 2006).

The physicochemical characteristics of volcanic particulate matter will determine some of health effects associated to exposures to volcanic eruptions. In general, volcanic and other forms of particulates, such as coal fly ash and urban particulates, contain a number of metals, including iron. In volcanic materials (ash),  $\text{Fe}^{3+}$  comprises just 10-15% of the total iron, while the majority is mainly  $\text{Fe}^{2+}$ . While iron is predominantly in the  $\text{Fe}^{2+}$  oxidation state in volcanic materials (most likely in the form of silicate glasses, silicate minerals, and FeTi-oxides), it will get oxidized as it is released into the atmosphere, so the iron will be present in the  $\text{Fe}^{3+}$  oxidation state in the leachates (Kelley and Cottrell 2009). As free iron levels are very low in biological fluids ( $< 10^{-18}$  M)(Bullen et al. 2005), particulates can be an exogenous iron source for bacteria. Furthermore, iron mobilization in coal fly ash is associated with aluminosilicate glass phases (Veranth et al. 2000), particle size (Chen et al. 2012)and iron speciation (Fu et al. 2012).

Therefore, total iron content is not enough to understand and predict the propensity of iron solubility in iron containing particles, such as volcanic ash.

In summary, this data suggests that exposure to respirable volcanic ash may increase the likelihood of developing bacterial infections via effects on both the bacteria and the innate immune system (Figure 5). The figure summarizes our findings, including the fact that in vitro exposure to ash from the Eyjafjallajökull volcano compromises alveolar macrophage function and anti-bacterial peptide activity. In addition, we found that the presence of volcanic ash led to increased growth of *Pseudomonas aeruginosa*. We suggest these data provide a new mechanistic paradigm for the adverse effects of volcanic ash exposure on respiratory health.

## REFERENCES

- Amaral AF, Rodrigues AS. 2007. Chronic exposure to volcanic environments and chronic bronchitis incidence in the azores, portugal. *Environ Res* 103:419-423.
- Baltrusaitis J, Usher CR, Grassian VH. 2007. Reactions of sulfur dioxide on calcium carbonate single crystal and particle surfaces at the adsorbed water carbonate interface. *Phys Chem Chem Phys* 9:3011-3024.
- Baxter PJ, Ing R, Falk H, French J, Stein GF, Bernstein RS, et al. 1981. Mount st helens eruptions, may 18 to june 12, 1980. An overview of the acute health impact. *JAMA : the journal of the American Medical Association* 246:2585-2589.
- Baxter PJ. 1983. Health hazards of volcanic eruptions. *J R Coll Physicians Lond* 17:180-182.
- Bernstein RS, Baxter PJ, Falk H, Ing R, Foster L, Frost F. 1986. Immediate public health concerns and actions in volcanic eruptions: Lessons from the mount st. Helens eruptions, may 18-october 18, 1980. *American Journal of Public Health* 76:25-37.
- Borisova AY, Toutain JP, Stefansson A, Gouy S, de Parseval P. 2012. Processes controlling the 2010 Eyjafjallajökull explosive eruption. *Journal of Geophysical Research – Solid Earth* 117:B05202, doi:10.1029/2012JB009213.
- Bullen JJ, Rogers HJ, Spalding PB, Ward CG. 2005. Iron and infection: The heart of the matter. *Fems Immunol Med Mic* 43:325-330.
- Caraballo JC, Yshii C, Westphal W, Moninger T, Comellas AP. 2011. Ambient particulate matter affects occludin distribution and increases alveolar transepithelial electrical conductance. *Respirology (Carlton, Vic)* 16:340-349.
- Caraballo JC, Borchering J, Thorne PS, Comellas AP. 2012. Pkc-zeta mediates diesel exhaust particles-induced lung injury. *American journal of respiratory cell and molecular biology*.
- Carlsen HK, Hauksdottir A, Valdimarsdottir UA, Gislason T, Einarsdottir G, Runolfsson H, et al. 2012. Health effects following the eyjafjallajökull volcanic eruption: A cohort study. *BMJ Open* 2:1-11.
- Carter AB, Knudtson KL, Monick MM, Hunninghake GW. 1999a. The p38 mitogen-activated protein kinase is required for nf-kappab- dependent gene expression. The role of tata-binding protein (tbp). *The Journal of biological chemistry* 274:30858-30863.

- Carter AB, Monick MM, Hunninghake GW. 1999b. Both erk and p38 kinases are necessary for cytokine gene transcription. *American journal of respiratory cell and molecular biology* 20:751-758.
- Chen H, Laskin A, Baltrusaitis J, Gorski CA, Scherer MM, Grassian VH. 2012. Coal fly ash as a source of iron in atmospheric dust. *Environ Sci Technol* 46:2112-2120.
- Convit J, Ulrich M, Castillo J, De Lima H, Perez M, Caballero N, et al. 2006. Inorganic particles in the skin of inhabitants of volcanic areas of central america: Their possible immunomodulatory influence in leishmaniasis and leprosy. *Trans R Soc Trop Med Hyg* 100:734-739.
- Dobbs LG, Gonzalez R, Williams MC. 1986. An improved method for isolating type ii cells in high yield and purity. *The American review of respiratory disease* 134:141-145.
- Dockery DW, Pope CA, 3rd, Xu X, Spengler JD, Ware JH, Fay ME, et al. 1993. An association between air pollution and mortality in six u.S. Cities. *The New England journal of medicine* 329:1753-1759.
- Elliott P, Shaddick G, Wakefield JC, de Hoogh C, Briggs DJ. 2007. Long-term associations of outdoor air pollution with mortality in great britain. *Thorax* 62:1088-1094.
- Fu H, Lin J, Shang G, Dong W, Grassian VH, Carmichael GR, et al. 2012. Solubility of iron from combustion source particles in acidic media linked to iron speciation. *Environ Sci Technol* 46:11119-11127.
- Gislason SR, Hassenkam T, Nedel S, Bovet N, Eiriksdottir ES, Alfredsson HA, et al. 2011. Characterization of eyjafjallajokull volcanic ash particles and a protocol for rapid risk assessment. *Proceedings of the National Academy of Sciences of the United States of America* 108:7307-7312.
- Grose EC, Grady MA, Illing JW, Daniels MJ, Selgrade MK, Hatch GE. 1985. Inhalation studies of mt. St. Helens volcanic ash in animals. Iii. Host defense mechanisms. *Environ Res* 37:84-92.
- Gudmundsson G. 2011. Respiratory health effects of volcanic ash with special reference to iceland. A review. *Clin Respir J* 5:2-9.
- Gudmundsson MT, Thordarson T, Hoskuldsson A, Larsen G, Bjornsson H, Prata FJ, et al. 2012. Ash generation and distribution from the april-may 2010 eruption of eyjafjallajokull, iceland. *Sci Rep* 2:572.

- Gutierrez MG, Master SS, Singh SB, Taylor GA, Colombo MI, Deretic V. 2004. Autophagy is a defense mechanism inhibiting bcg and mycobacterium tuberculosis survival in infected macrophages. *Cell* 119:753-766.
- Hansell AL, Horwell CJ, Oppenheimer C. 2006. The health hazards of volcanoes and geothermal areas. *Occup Environ Med* 63:149-156, 125.
- Karp PH, Moninger TO, Weber SP, Nesselhauf TS, Launspach JL, Zabner J, et al. 2002. An in vitro model of differentiated human airway epithelia. *Methods for establishing primary cultures. Methods Mol Biol* 188:115-137.
- Kelley KA, Cottrell E. 2009. Water and the oxidation state of subduction zone magmas. *Science (New York, NY)* 325:605-607.
- Klein-Patel ME, Diamond G, Boniotto M, Saad S, Ryan LK. 2006. Inhibition of beta-defensin gene expression in airway epithelial cells by low doses of residual oil fly ash is mediated by vanadium. *Toxicological sciences : an official journal of the Society of Toxicology* 92:115-125.
- Lave LB, Seskin EP. 1972. Air pollution, climate, and home heating: Their effects on u.S. Mortality rates. *American Journal of Public Health* 62:909-916.
- Longo BM, Yang W. 2008. Acute bronchitis and volcanic air pollution: A community-based cohort study at kilauea volcano, hawai'i, USA. *J Toxicol Environ Health A* 71:1565-1571.
- Martin TR, Wehner AP, Butler J. 1986. Evaluation of physical health effects due to volcanic hazards: The use of experimental systems to estimate the pulmonary toxicity of volcanic ash. *American Journal of Public Health* 76:59-65.
- Monick MM, Carter AB, Gudmundsson G, Mallampalli R, Powers LS, Hunninghake GW. 1999. A phosphatidylcholine-specific phospholipase c regulates activation of p42/44 mitogen-activated protein kinases in lipopolysaccharide- stimulated human alveolar macrophages. *J Immunol* 162:3005-3012.
- Monick MM, Powers LS, Gross TJ, Flaherty DM, Barrett CW, Hunninghake GW. 2006. Active erk contributes to protein translation by preventing jnk-dependent inhibition of protein phosphatase 1. *J Immunol* 177:1636-1645.
- Monick MM, Powers LS, Barrett CW, Hinde S, Ashare A, Groskreutz DJ, et al. 2008. Constitutive erk mapk activity regulates macrophage atp production and mitochondrial integrity. *J Immunol* 180:7485-7496.

- Monick MM, Powers LS, Walters K, Lovan N, Zhang M, Gerke A, et al. 2010. Identification of an autophagy defect in smokers' alveolar macrophages. *J Immunol* 185:5425-5435.
- Naumova EN, Yepes H, Griffiths JK, Sempertegui F, Khurana G, Jagai JS, et al. 2007. Emergency room visits for respiratory conditions in children increased after guagua pichincha volcanic eruptions in april 2000 in quito, ecuador observational study: Time series analysis. *Environ Health* 6:21.
- Olgun N, Duggen S, Croot PL, Delmelle P, Dietze H, Schacht U, et al. 2011. Surface ocean iron fertilization: The role of subduction zone and hotspot volcanic ash and fluxes into the pacific ocean. *Global Biogeochemical Cycles* 25:GB4001.
- Peate DW, Breddam K, Baker JA, Kurz MD, Barker AK, Prestvik T, et al. 2010. Compositional characteristics and spatial distribution of enriched icelandic mantle components. *J Petrol* 51:1447-1475.
- Petecchia L, Sabatini F, Varesio L, Camoirano A, Usai C, Pezzolo A, et al. 2009. Bronchial airway epithelial cell damage following exposure to cigarette smoke includes disassembly of tight junction components mediated by the extracellular signal-regulated kinase 1/2 pathway. *Chest* 135:1502-1512.
- Philibert RA, Sears RA, Powers LS, Nash E, Bair T, Gerke AK, et al. 2012. Coordinated DNA methylation and gene expression changes in smoker alveolar macrophages: Specific effects on vegf receptor 1 expression. *Journal of leukocyte biology* 92:621-631.
- Reisetter AC, Stebounova LV, Baltrusaitis J, Powers L, Gupta A, Grassian VH, et al. 2011. Induction of inflammasome-dependent pyroptosis by carbon black nanoparticles. *The Journal of biological chemistry* 286:21844-21852.
- Roberts JR, Young SH, Castranova V, Antonini JM. 2009. The soluble nickel component of residual oil fly ash alters pulmonary host defense in rats. *J Immunotoxicol* 6:49-61.
- Sabatini DD, Bensch K, Barnett RJ. 1963. Cytochemistry and electron microscopy. The preservation of cellular ultrastructure and enzymatic activity by aldehyde fixation. *The Journal of cell biology* 17:19-58.
- Samet JM, Dominici F, Curriero FC, Coursac I, Zeger SL. 2000. Fine particulate air pollution and mortality in 20 u.S. Cities, 1987-1994. *The New England journal of medicine* 343:1742-1749.

- Schmidt A, Ostro B, Carslaw KS, Wilson M, Thordarson T, Mann GW, et al. 2011. Excess mortality in Europe following a future Iaki-style Icelandic eruption. *Proceedings of the National Academy of Sciences of the United States of America* 108:15710-15715.
- Sigmarrsson O, Vlastelic I, Andreasen R, Bindeman I, Devidal J-L, Moune S, et al. 2011. Remobilization of silicic intrusion by mafic magmas during the 2010 Eyjafjallajökull eruption. *Solid Earth* 2:271-281. doi:10.5194/se-2-271-2011.
- Sigmundsson F, Hreinsdóttir S, Hooper A, Arnadóttir T, Pedersen R, Roberts MJ, et al. 2010. Intrusion triggering of the 2010 Eyjafjallajökull explosive eruption. *Nature* 468:426-430.
- Slebos DJ, Ryter SW, van der Toorn M, Liu F, Guo F, Baty CJ, et al. 2007. Mitochondrial localization and function of heme oxygenase-1 in cigarette smoke-induced cell death. *American journal of respiratory cell and molecular biology* 36:409-417.
- Soberanes S, Urich D, Baker CM, Burgess Z, Chiarella SE, Bell EL, et al. 2009. Mitochondrial complex III-generated oxidants activate Ask1 and JNK to induce alveolar epithelial cell death following exposure to particulate matter air pollution. *The Journal of biological chemistry* 284:2176-2186.
- Spix C, Heinrich J, Dockery D, Schwartz J, Volksch G, Schwinkowski K, et al. 1993. Air pollution and daily mortality in Erfurt, East Germany, 1980-1989. *Environmental Health Perspectives* 101:518-526.
- Swindles GT, Lawson IT, Savov IP, Connor CB, Plunkett G. 2011. A 7000 yr perspective on volcanic ash clouds affecting Northern Europe. *Geology* 39:887-890.
- Thorsteinsson T. 2012. High levels of particulate matter in Iceland due to direct ash emissions by the Eyjafjallajökull eruption and resuspension of deposited ash. *Journal of Geophysical Research – Solid Earth* 117:1-9.
- Upadhyay D, Panduri V, Ghio A, Kamp DW. 2003. Particulate matter induces alveolar epithelial cell DNA damage and apoptosis: Role of free radicals and the mitochondria. *American journal of respiratory cell and molecular biology* 29:180-187.
- Veranth JM, Smith KR, Huggins F, Hu AA, Lighty JS, Aust AE. 2000. Mossbauer spectroscopy indicates that iron in an aluminosilicate glass phase is the source of the bioavailable iron from coal fly ash. *Chem Res Toxicol* 13:161-164.
- Wiesner J, Vilcinskas A. 2010. Antimicrobial peptides: The ancient arm of the human immune system. *Virulence* 1:440-464.

Witham CS, Oppenheimer C, Horwell CJ. 2005. Volcanic ash-leachates: A review and recommendations for sampling methods. . J Volcanol Geoth Res 141, :299-326.

## Figure legends

**Figure 1.** SEM of lung epithelial cells and alveolar macrophages exposed to volcanic ash. A. Three images at different magnifications of a single cell isolation show rat lung epithelial cells exposed to  $20 \mu\text{g}/\text{cm}^2$  volcanic ash. At three hours, cells were fixed and SEM performed. Black arrows point out one of several ash particles in image. White arrows point to microvilli on alveolar epithelial cells. B. Human alveolar macrophages internalize volcanic ash. Human alveolar macrophages were exposed to  $20 \mu\text{g}/\text{cm}^2$  volcanic ash. At three hours, cells were fixed and SEM performed. C. Alveolar macrophages were exposed to volcanic ash ( $2 \mu\text{g}/\text{cm}^2$ ) for 3 hours. Cells were fixed and prepared for transmission electron microscopy (TEM). Image is one of many showing ash inside vesicles in the cytosol of exposed macrophage. D. Scanning transmission electron microscopy (STEM)/high angle annular dark field (HAADF) and energy-dispersive spectrometry (EDS) analysis was performed on human alveolar macrophages exposed to volcanic ash ( $2 \mu\text{g}/\text{cm}^2$ ) for three hours. STEM/EDS elemental analysis shows that particles are rich in elements, typical to those of volcanic ash. Asterisks show osmium peaks from thin section staining. Elemental analysis confirmed by EDS analysis.

**Figure 2.** Volcanic ash exposure alters macrophage function. A. Human alveolar macrophages were cultured in the presence of ash ( $20 \mu\text{g}/\text{cm}^2$ , A, B and C ashes described in methods) for 5 hours. Whole cell proteins were isolated and Western analysis performed. LC3B-II levels show an accumulation of autophagosomes. Increased ubiquitin conjugates are demonstrated at all molecular weights. Shown are representative blots from three experiments. B. Human alveolar macrophages were exposed to volcanic ash ( $20 \mu\text{g}/\text{cm}^2$ ) for 30 minutes prior to LPS (100 ng/ml) exposure for 30 minutes. Western analysis of phosphorylated (activated MAP kinases show a

decrease in LPS activation with ash exposure. Shown are representative blots from three experiments. C. Human alveolar macrophages were exposed to volcanic ash ( $20 \mu\text{g}/\text{cm}^2$ ) for 30 minutes prior to LPS ( $100 \text{ ng}/\text{ml}$ ) exposure for 3 hours. RNA was isolated and TNF $\alpha$  mRNA measured by Realtime RT-PCR ( $n=3$ ). Data represent mean  $\pm$  standard error of the mean (SE). D. The presence of ash decreases bacterial killing (*Pseudomonas*) by alveolar macrophages. PAO1 phagocytosis was determined with CFU counting after 20 minutes exposure. Isolated rat macrophages pre-exposed to ash for 2 hours had phagocytosed similar amounts of ash ( $N=4$  in triplicates). Data represent mean  $\pm$  SE. E. Bacterial killing was determined by analyzing difference between CFU engulfment at 110 and 20 minutes. PAO1 killing in the presence of ash was inhibited, although not statistically significant ( $p=0.07$ ,  $N=4$  in triplicates). Data represent mean  $\pm$  SE.

**Figure 3.** Icelandic ash exposure does not alter alveolar epithelium electrical resistance or viability. A. Isolated rat alveolar epithelial cells were exposed to 10, 50 or  $100 \mu\text{g}/\text{cm}^2$  of sieved volcanic ash.  $100 \mu\text{g}/\text{cm}^2$  did not significantly increase cell death (propidium iodide staining) or decrease cell viability (trypan blue). Transepithelial electrical conductance ( $Gt$ ) at 10 and  $50 \mu\text{g}/\text{cm}^2$  was also not significantly increased ( $N=3$  in triplicates). Data represent mean  $\pm$  SE. B. Isolated human airway epithelial cells were exposed to 10, 50, and  $100 \mu\text{g}/\text{cm}^2$  sieved ash. At  $100 \mu\text{g}/\text{cm}^2$ , cell death was not increased as measured by propidium iodide (left) ( $N=2$  in triplicates). At 10 and  $50 \mu\text{g}/\text{cm}^2$ ,  $Gt$  was not significantly increased ( $N=3$  in triplicates). Data represent mean  $\pm$  SE.

**Figure 4.** Volcanic ash alters bacterial growth and bacterial killing by anti-microbial peptides. A. PAO1 growth was observed by measuring  $\text{OD}_{600}$  for nine hours.  $\text{FeCl}_3$  and  $\text{Al}_2\text{O}_3$  were used respectively as a soluble source of iron and control for particle effects. Ash increased

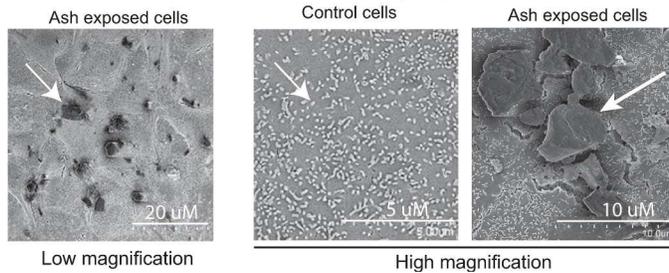
PAO1 growth when compared with control (\* $p < 0.0001$  at 9 hours, calculated using Student's t test) (N=3 in triplicates). B. Diagram of antimicrobial peptide activity assay, lysogeny broth (LB).C. Ash was exposed to a cocktail of lysozyme, lactoferrin and  $\beta$ -defensins 1 and 2 and killing capacity was determined by  $OD_{600}$  measurement after PAO1 exposure. ( $p < 0.0001$ , calculated using Student's t test) (N=3 in triplicates).

**Figure 5.** Diagram of findings on effects of volcanic ash (Eyjafjallajökull, 2010) in innate immune system and bacterial growth.

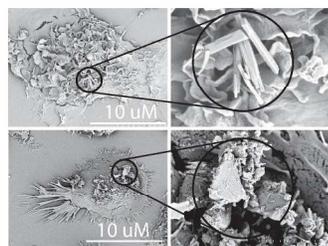
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Figure 1

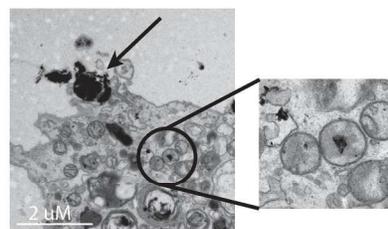
A. Rat alveolar epithelial cells exposed to Icelandic ash (SEM)



B. Human alveolar macrophages with Icelandic ash (SEM)



C. Human alveolar macrophages exposed to Icelandic ash (TEM)



D. STEM/HAADF image of alveolar macrophages (heavier elements appear brighter).

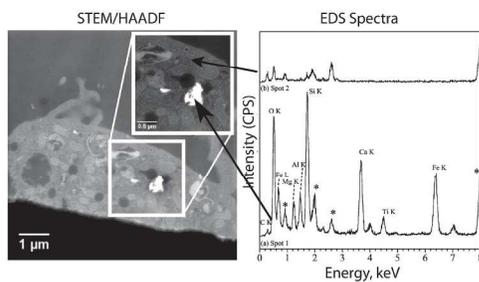


Figure 1  
243x300mm (300 x 300 DPI)

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Figure 2

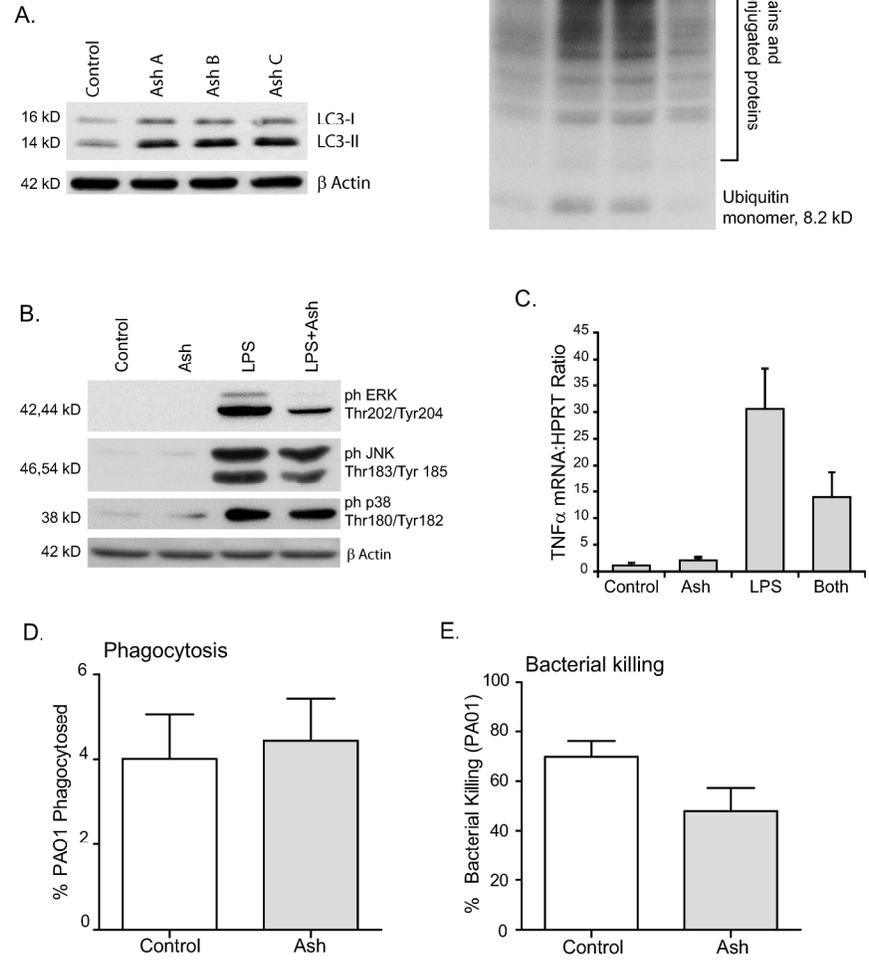


Figure 2  
221x309mm (300 x 300 DPI)

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2012

Figure 3

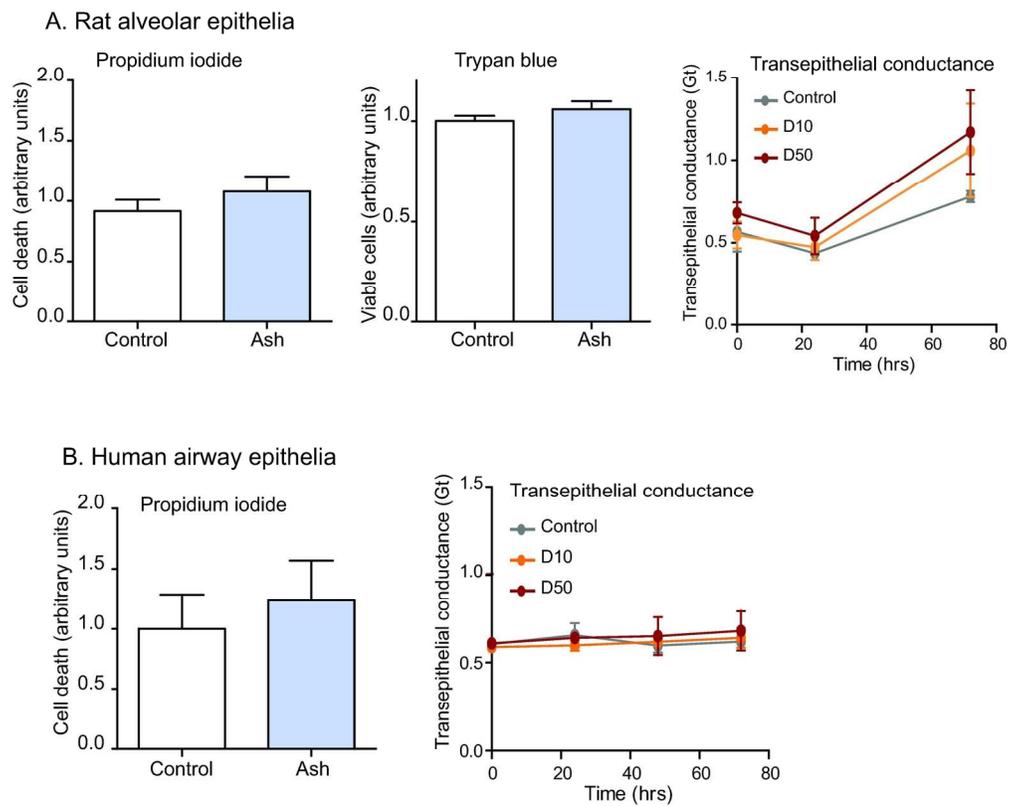
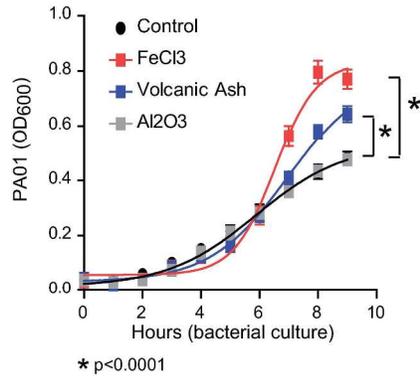


Figure 3  
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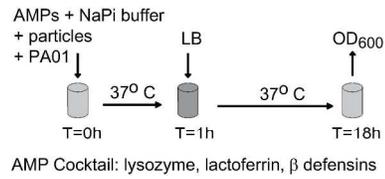
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Figure 4

A. Bacterial growth (PA01)



B. Antimicrobial peptide activity assay



C. Antimicrobial peptide activity assay

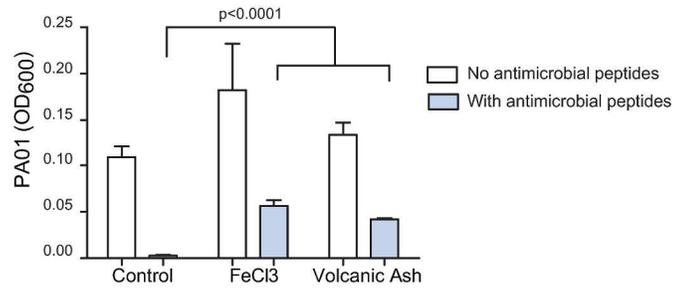


Figure 4  
221x384mm (300 x 300 DPI)

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Figure 5

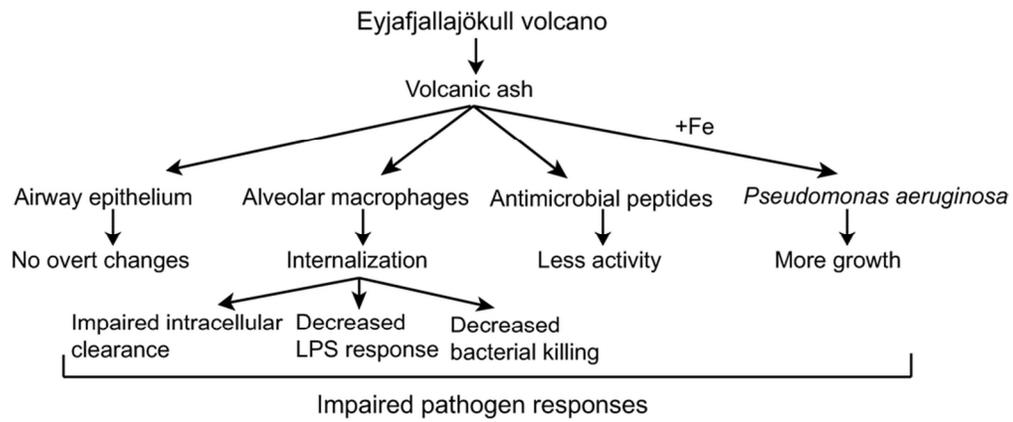


Figure 5  
87x49mm (300 x 300 DPI)