Organic components of airborne dust influence the magnitude and kinetics of dendritic cell activation

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Abstract

Bioaerosol exposure in highly contaminated occupational settings is associated with an increased risk of disease. Yet, few determinants allow for accurate prediction of the immunopathogenic potential of complex bioaerosols. Since dendritic cells are instrumental to the initiation of immunopathological reactions, we studied how dendritic cell activation was modified in response to individual agents, combined microbial agents, or air sample eluates from highly contaminated environmental settings. We found that combinations of agents accelerated and enhanced the activation of in vitro-generated murine bone marrow-derived dendritic cell cultures, when compared to individual agents. We also determined that endotoxins are not sufficient to predict the potential of air samples to induce bone marrow-derived dendritic cell activation, especially when endotoxin levels are low. Importantly, bone marrow-derived dendritic cell activation stratified samples from three environmental settings (swine barns, dairy barns, and wastewater treatment plants) according to their air quality status. As a whole, these results support the notion that the interplay between bioaerosol components impacts on their ability to activate dendritic cells and that bone marrow-derived dendritic cell cultures are promising tools to study the immunomodulatory impact of air samples and their components.

1. Introduction

Environments heavily contaminated with bioaerosols are associated with increased prevalence of adverse symptoms and diseases (Greskevitch et al., 2007; May et al., 2012). Bioaerosols are aerosolized particles that may be composed of microorganisms and fragments of plants, animals and soil. The diversity of contemporary activities leading to the generation of bioaerosol is increasing, and the nature of the immunopathogenic stimuli likely differs between occupational settings. For instance, swine confinement buildings (Nehme et al., 2008), dairy barns (Blais Lecours et al., 2012) and wastewater treatment plants (Bauer et al., 2002) display different concentrations of airborne organic dust (Kirkhorn and Garry, 2000), bacteria (Blais-Lecours et al., 2015), archaea (Nehme et al., 2009), fungi (Eduard et al., 2012) and endotoxins (Mayeux, 1997). Although general patterns of bioaerosols are found among different occupational settings, predicting their propensity to elicit immunopathological responses remains a challenge.

The nature of the interactions between components found in bioaerosols *versus* their impact on human health remains misunderstood. Several studies have attempted to correlate single parameters, such as endotoxins or total organic dust content, with the occurrence of symptoms (Donham et al., 2000; Vogelzang et al., 1997). Together, these studies failed to agree on an existing and significant correlation (May et al., 2012). This lack of consensus has led, among others, to the concept that combination of bioaerosol components can have additive, synergistic, or even antagonistic effects (Liebers et al., 2008). Yet, these hypotheses still need to be confirmed.

Dendritic cells (DCs) are located at the interface of the host and his environment and they play a central role in the initiation of immune reactions (Vermaelen and Pauwels, 2005). These cells express a wide variety of pattern-recognition receptors and are highly sensitive to a wide array of immunogenic components (Banchereau and Steinman, 1998). Upon encounter with various agents including endotoxins, peptidoglycans, zymosan, poly-(I:C), Pam_3Cys-Ser-$(Lys)_4$, polyuridylic acid, CpG and β-D glucans (Dearman et al., 2009), DCs initiate an activation process in order to fulfill their role of professional antigen presenting cells. This process, which is replicated in vitro by murine bone marrow-derived dendritic cell (BMDC) cultures, involves the cell surface upregulation of the major histocompatibility complex-II (MHC II) and co-stimulatory molecules in order to effciently prime cells of the acquired arm of the immune system. DCs also represent sources of pro-inflammatory cytokines, and consequently play key functions in the amplification of the innate and acquired immune responses (Banchereau and Steinman, 1998).

In line with these functions, the intensity of immune responses was repeatedly shown to correlate with the level of DC activation *in vivo* (Blais Lecours et al., 2011; Marsolais et al., 2009). In addition to the magnitude of the DC responses, other parameters could also influence the nature of the reactions triggered by foreign particles. For instance, the kinetics of DC activation can influence the polarity of immunity with early-activated DCs having a T_H1-polarizing phenotype, while late-activated DCs being biased towards T_H2 polarity (Langenkamp et al., 2000). Although antigenic load can influence the magnitude and kinetics of DC activation (Langenkamp et al., 2000), the stimulatory impact of complex antigens, as they are found in bioaerosols, remains mischaracterized.

The primary goal of this study was to determine if the complexity of antigenic triggers influenced the kinetics and the magnitude of BMDC activation *in vitro*. We also investigated which parameter accounted for BMDC activation by air sample eluates (ASE) obtained from three distinct occupational settings. We found that combinations of different stimuli accelerated BMDC activation and enhanced the absolute level of active BMDCs. Moreover, air samples from three different working environments displayed a differential potency to induce BMDC activation, which was consistent with their respective air quality status.

2. Materials and methods

2.1. BMDC culture and stimulation

Bone marrow cells were harvested from the hind limbs of C57BL/6J mice and cultured in presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) in order to generate large amounts of immature BMDCs, as described (Inaba et al., 1992; Lutz et al., 1999), with minor modifications (supplementary methods). Bone marrow cells were seeded into Petri dishes at a concentration of 5×10^6 cells. After 7 days of culture with GM-CSF, the non-adherent cells were seeded in a 24-well plate at a concentration of 3×10^5 cells per well. These enriched immature BMDC cultures were then incubated with microbial components (see supplementary methods for details) or with ASE, for up to 24 h. Supernatants were collected and stored at −80 °C and cells were harvested using trypsin-EDTA.

2.2. Flow cytometric analyses

BMDCs were labeled using the following commercially-available reagents: PE anti-CD11c (N418; Biolegend, San Diego, CA), PE-Cy7 anti-CD11b (M1/70; BD Bioscience, San Jose, CA), Pacific Blue anti-MHC II (M5/114.15.2; Biolegend), biotin anti-CD86 (GL1; BD pharmingen, San Diego, CA). Streptavidin-Alexa Fluor 700 (Invivogen) was used as a second step reagent. Data were collected with FACS Diva-driven LSR Fortessa (Becton Dickinson) and analyzed with the FlowJo software (Tree star, Inc., San Carlos, CA). After positive selection of the whole $CD11c^+$ $CD11b^+$ BMDC population, the frequency of CD86hi MHCIIhi BMDCs was used as a surrogate for activation (Fig. 1A). Fluorescence minus one (FMO) controls were performed at every experiment. When multiple experiments were pooled, data were normalized to an internal control (100 ng/ml endotoxins, Figs. 1, 4, 5). Time-course experiments (Fig. 2) show raw frequencies of $CD86^{hi}$ MHC II^{hi} BMDCs out of the total viable BMDC pool.

2.3. Enzyme-linked immunosorbent assay (ELISA)

TNF was quantified in supernatants from BMDC cultures using the Mouse TNF-α ELISA MAX™ Standard (Biolegend).

2.4. Collection and processing of air samples

Samples from 9 swine barns, 3 dairy barns and 3 wastewater treatment plants were collected with the SASS® 3100 Dry Air Sampler (Research International, Monroe, WA) at 300 l per minute (lpm) for 10 min. The electret filters were eluted with phosphate-buffer saline (PBS) (pH 7.4) with the SASS® 3010 Particle Extractor (Research International) in order to obtain ASE an equivalent of 600 l of sampled air per ml. This procedure allowed efficient recovery of endotoxins in a medium compatible with cell culture (Fig. S1). One aliquot was centrifuged at 21000 ×g for 10 min and the pellet was used for total DNA extraction. Other aliquots were kept at −20 °C until further use.

2.5. Endotoxin and total organic dust quantification

The kinetic chromogenic limulus amebocyte lysate (LAL) assay (LONZA, Walkersville, MD) was used to quantify endotoxins according to the manufacturer's instruction. A β-1,3-glucan blocker was used to insure specificity for endotoxins (LONZA). Total organic dust was quantified on field with the DustTrak™ DRX Aerosol Monitor Model 8534 (TSI, St Paul, MN).

2.6. Total DNA extraction

Powerlyzer Powersoil DNA extraction kit (MO BIO, Carlsbad, CA) was used to obtain total DNA from air samples using the manufacturer's protocol. A bead-beating step was performed with the Mixer Mill MM300 (Retsch, Haan, Germany) at a speed of 20 Hz for 20 min after a heating step at 70 °C for 10 min. DNA extracts were stored at −20 °C until analyses.

2.7. Quantitative real-time polymerase chain reaction (qPCR)

PCR quantifications were done using CFX-96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA). Data were analyzed using the Bio-Rad CFX Manager software (Version 3.1). Total bacterial 16S rRNA gene quantification was performed using primers (RP 16S rDNA and FP 16S rDNA) and probe (probe 16S rDNA), as described (Bach et al., 2002), using iQ Supermix (Bio-rad). The amplification program was the following: one step at 95 °C for 3 min, followed by 40 cycles at 95 °C for 20 s and 62 °C for 60 s before fluorescence acquisition. A ten-fold dilution of *Escherichia coli* DNA was used for standard curve and results were converted to total bacteria 16S rRNA gene. Total achaeal 16S rRNA gene quantification was performed using A751F and A976R (Baker et al., 2003; Reysenbach and Pace, 1995) primers as described (Blais Lecours et al., 2012). A ten-fold dilution of the genomic DNA of *Methanobrevibacter smithii* (MBS) was also used as a standard curve. Quantification of mtaB1 gene of *Methanosphaera stadtmanae* (MSS) was performed using primers as described (Blais Lecours et al., 2014). The standard curve consisted in ten-fold MSS DNA serial dilutions. The quantification of the *NifH* gene of MBS was performed using primers and probe as previously described (Johnston et al., 2010). A ten-fold MBS genomic DNA dilution was used as standard curve.

2.8. Statistical analyses

The variables of combined stimulations were analyzed using a one-way analysis of variance (ANOVA) to compare groups. The normality assumption was verified with the Shapiro-Wilk test on residuals from the statistical model. The Brown and Forsythe's variation of Levene's test statistic was used to verify the homogeneity of variances. The relationship between BMDC activation and the individual components of air samples was described using Pearson correlation. All data followed a log-normal distribution and were log-transformed before statistical analysis.

Predictors of BMDC activation were found using regression models based on p-values or Akaike information criterion. The impact of endotoxins was also analyzed on selected variables after performing a dichotomization of the endotoxin variable using the Ward classification method. Normality hypothesis was verified using Shapiro-Wilk statistic. The homogeneity of variances was visually checked with residual plots.

Analyses were conducted using the statistical package SAS, version 9.4 (SAS Institute Inc., Cary, NC) and R (R Core Team (2016), Vienna, Austria.) by a biostatistician. All data are expressed using means ± standard errors of the mean (SEM) and significance was set at $p \leq 0.05$.

3. Results

3.1. Differing impact of bioaerosol components on dendritic cell activation

The potential of BMDCs to discriminate between immunogenic bioaerosol components was evaluated using isolated agents frequently found in bioaerosols (Fig. 1). The maximal BMDC activation response to endotoxins was approximately twice of that induced by peptidoglycans (PGN), and β-D glucans (Fig. 1B). Whole microorganisms also diferentially activated BMDCs (Fig. 1C). At the concentration of 10^4 ng/ml, the Gram-positive *Bacillus atrophaeus* and the Gram-negative *Pseudomonas aeruginosa* induced a relative BMDC activation of 87.7 ± 6.0% and 102.5 ± 6.2%, respectively. On the other hand, *Saccharopolyspora rectivirgula* and archaeal species MSS and MBS were less potent, inducing a maximal relative BMDC activation of 48.7 ± 10.2%, 43.1 ± 5.3% and 17.2 ± 7.8%, respectively.

3.2. Combination of agents quantitatively and qualitatively influences BMDC activation

Dose-response curves of endotoxins were performed in presence of different concentrations of MSS (Fig. 1D) or PGN (Fig. 1E). We observed that concentrations of MSS or PGN sufficient to induce a relative BMDC activation of approximately 60% (10 µg/ml) displayed an additive effect on BMDC activation at the lowest endotoxin concentration tested. Moreover, the combination of either MSS or PGN (10 µg/ml) with endotoxins always resulted in higher BMDC activation, regardless of the endotoxin concentration. These results suggest that combinations of agents can act additively on BMDC activation and increase the overall magnitude of the response.

Given the additive effects of combined agents, and since the kinetics of DC activation can influence the nature of the ensuing response (Langenkamp et al., 2000), we determined if combinations of agents influenced the rate of BMDC activation (Fig. 2). We found that the absolute percentage of active BMDCs was increased by MSS (Fig. 2A-B) or PGN (Fig. 2C-D) for all endotoxin concentrations tested. Moreover, this

enhancement was detected at earlier time points, when compared to incubations with single agents. Indeed, the absolute percentage of active BMDCs obtained in response to MSS 10 μg/ml and endotoxins at 8 h was always higher than the absolute percentage of activated BMDCs induced by either 10 μg/ml of MSS or endotoxins alone. A similar enhancement was observed at 12 h of incubation with PGN 10 μg/ml and 0.1 or 1 ng/ml of endotoxins. As a whole, these results support that the complexity of bioaerosols not only affects the magnitude of the activation response, but also its kinetics.

3.3. Relative BMDC activation stratifies environmental samples according to their air quality status

Characterization of environmental samples confirmed the heavier bioaerosol burden of swine barns, compared to dairy barns and was-tewater treatment plants. Indeed, swine barns featured the highest endotoxin concentrations ranging from 6.7 to 1696.5 EU/m^3 of air (Fig. 3A) and an average total organic dust of 2.7 ± 2.2 mg/m^3 (Fig. 3B). They were also highly contaminated with total bacteria (8.6 × 10^7 to 2.3 × 10^9 16S rRNA gene/m^3 of air) (Fig. 3C) and total archaea (1.3 × 10^6 to 3.1 × 10^8 16S rRNA gene/m^3)(Fig. 3D). Two species of archaea found in bioaerosols were quantified by PCR because of their immunogenic potential (Blais Lecours et al., 2011). While MBS was commonly found in these facilities (4.2 × 10^4 to 9.5 × 10^6 *NifH* gene copy/m^3 of air) (Fig. 3E), MSS was only detected in three swine barns (0.2 × 10^6 to 3.8 × 10^6 *MtaB1* gene/m^3 of air) (Fig. 3F). The air of dairy barns featured lower endotoxin concentrations (0.1 to 8.2 EU/m^3 of air) and total organic dust (0.2 to 0.6 mg/m^3 of air) compared to swine barns. They also showed lower concentrations of total bacteria (1.8 × 10^6 to 1.2 × 10^7 16S rRNA gene/m^3 of air) and total archaea (0.05 × 10^6 and 0.1 × 10^6 16S archaeal rRNA gene/m^3) and MBS was found in one dairy barn (9.8 × 10^3 *NifH* gene/m^3). Wastewater treatment plants featured low concentrations of endotoxins (0.2 to 5.3 EU/m^3)(Fig. 3A) and total bacteria (0.03 × 10^6 to 0.2 × 10^6 16S rRNA gene/m^3 of air) (Fig. 3C).

The impact of these environmental samples on BMDC activation was then assessed (Fig. 4). Swine barns were the most potent environments tested and induced a relative BMDC activation of 84.1 to 126.9% at the 0.1 m^3 ASE concentration. Under the same experimental conditions, dairy barns induced relative BMDC activation ranging from 35.5 to 72.7%; and wastewater treatment plants from 3.0 to 21.1% (Fig. 4A). TNF levels (Fig. 4B) paralleled the findings made with BMDC activation. For instance, an incubation with 0.1 m^3 of ASE from swine barns generated a TNF secretion ranging from 183.6 to 839.8%, whereas those from dairy barns and wastewater treatment plants where considerably lower (from 44.9 to 79.9% and 0.2 to 14.8% of the 100 ng/ml endotoxin standard, respectively). These results support the contention that BMDC activation can stratify occupational settings according to their expected air quality status.

3.4. Endotoxin content underestimates the potential of BMDC activation by complex samples

Endotoxins (Fig. 5A), total bacteria (Fig. 5B), total organic dust (Fig. 5C) and total archaea (Fig. 5D) displayed the strongest correlations with BMDC activation at 0.1 m^3 of ASE (r = 0.83, 0.93, 0.87 and 0.86, respectively). Yet, for a majority of samples, the ability to induce BMDC activation (Fig. 5A) was superior to their endogenous endotoxin content. The two major predictive parameters for BMDC activation were the total organic dust and endotoxin content, which explained 75% and 8% of BMDC activation, respectively. Moreover, our results show that samples with subthreshold levels of endotoxins (WTP3, DB3 and DB2) retain the ability to induce BMDC activation. If the endotoxin variable is dichotomized for low (< 10 EU/m^3) vs high (≥10 EU/m^3) content, then the total organic dust predictive factor on BMDC activation increased to 95% for the low content group, supporting the concept that alternative components should be considered as immunogenic agents, especially when endotoxins are underrepresented.

4. Discussion

Occupational settings featuring high bioaerosol content are associated with an increased risk of developing respiratory diseases. The pathognomonic mechanisms of non-infectious bioaerosol components are misunderstood, resulting in the absence of clear determinants of air quality. Our study unravels a complex interplay between antigenic triggers that not only modulates the magnitude of DC activation, but also its rate, which can impact on the nature of the immune response. As seen in working environments, we found that endotoxins are not sufficient to explain the impact of complex environmental samples on DC activation and this effect is magnified in environments where endotoxins are underrepresented. Importantly, we also determined that BMDC activation efficiently stratifies the different types of occupational settings included in this study according to their air quality status.

Organic dust is composed of a multitude of components proposed to have synergistic effects on the immune response. Yet, the mechanisms of synergy remain poorly understood. Our results support, on one hand, an additive effect of individual components regarding the magnitude of BMDC activation. Indeed, high doses of MSS or PGN and 0.1 ng/ml of endotoxins produced an additive BMDC activation. Moreover, sub-threshold doses of MSS or PGN didn't modify BMDC activation in response to endotoxins, somehow refuting the notion of synergy between these agents regarding the absolute level of activated BMDCs. On the other hand, our study reveals that the modulation of the rate of BMDC activation could mitigate the immunogenic potential of complex bioaerosols. Indeed, we found that specific combinations of agents differentially accelerated the activation of BMDCs. Critically, early-activated DCs display a T_H1-polarizing phenotype which has classically been associated with a neutrophilic/tissue damaging response (Kolaczkowska and Kubes, 2013); while lately-activated DCs might favour T_H2 polarity (Langenkamp et al., 2000) that is often associated to the release of IL-4 and by the accumulation of eosinophils (Humbles et al., 2004). Of note, the fact that combination of MSS to endotoxins was associated with a faster peak of BMDC activation, compared to the combination of PGN and endotoxins, is in line with previous *in vivo* observations that MSS induces a cytokine response that is dominated by TH1/TH17 cytokines (Bernatchez et al., 2017; Blais Lecours et al., 2011), while PGN has often been associated with the induction of a TH2 response (Matsui and Ikeda, 2014; Matsui and Nishikawa, 2012). Thus, our

findings support that the modulation of the rate of DC activation is an additional parameter contributing to the immunopathogenic impact of complex bioaerosols.

Our study supports the concept that BMDC activation can stratify the air quality status of different groups of occupational settings, which in this case, are all classified among the high bacterial concentration and high total organic dust category (Blais-Lecours et al., 2015; Thorne et al., 2016). Our results suggest that the primary immunogenic predictor of these environments is total organic dust. Such finding is intuitive given that the total organic dust parameter is a likely surrogate for the whole repertoire of immunogenic particles. In regards to the weaker predictive power of endotoxins regarding BMDC activation, one should also consider that multiple variants of the immunogenic lipid A portion of the endotoxins exist (Albiger et al., 2007), which can influence the pathogenicity of the bacterial strain (Backhed et al., 2003). Thus, the measure of endotoxins by the LAL assay may not effectively predict its immunological activity (Luchi and Morrison, 2000). Nevertheless, our findings underscore the sensitivity of BMDCs to the total charge of bioaerosols in a given environment; and suggest that such in vitro assay combined with a detailed characterization of bioaerosol components might lead to the identification of novel environment-specific markers of immunogenicity.

The generation of ASE suitable for *in vitro* assays has been challenging and our study also reveals a promising method to obtain samples in a physiological buffer. We had initially tested the high debit Coriolis µ sampler, but the use of detergents for sample retrieval from the plastic cuvettes revealed to be incompatible with our BMDC assay. In fact, we found that concentrations as low as 10^{-4}% Triton X-100 caused severe mortality over a 24 h incubation period (not shown). Fortunately, we determined that SASS® 3100 filters could be eluted with detergent-free buffer and retain effciency to sample endotoxins (Fig. S1). Still, Viegas et al. (Viegas et al., 2017) documented differential *in vitro* cytotoxic and pro-inflammatory impacts of environmental samples collected with the Coriolis µ/detergent procedure. Of note, their assay featured an 18 h incubation period along with a different cell type (THP-1 monocyte-like immortalized cells). Although their results also suggest that total organic dust might play an important role for modulation of cellular outcomes *in vitro*, it becomes difficult to reconcile the results of both studies given that endotoxins bind to plastic cuvettes (Fig. S1A) (AssociatesofCapeCod, 1988; Novitsky et al., 1986) and that endotoxins were not quantified in their study. Thus, our study supports the validity of SASS® 3100-derived samples eluted in PBS along with their compatibility with prolonged in vitro cell-based assays.

In fact, several cellular approaches were proposed to evaluate the inflammatory potential of bioaerosol components, including the whole blood assay and commercially-available cell lines. Of note, a number of findings also support the sensitivity of DCs to detect agents with differing immunogenic properties including bovine milk proteins (Davies et al., 2005), endotoxin mutants (Liu et al., 2009) and a multitude of toll-like receptors ligands (Dearman et al., 2009). We found the sensitivity of BMDCs to be comparable or superior to previously-documented cell-based assays used to gauge the immunogenicity of bioaerosol components. For instance, the epithelial cell line A549 requires 100 µg/ml of endotoxins in order to release significant amounts of IL-8 (Letourneau et al., 2010); and the release of reactive oxygen species (Timm et al., 2009) can be detected in granulocytes at 0.1 ng/ml of endotoxins. As for the whole blood assay, it can detect as low as 10 pg/ml of endotoxins (Punsmann et al., 2013), which is only one order of magnitude lower than what we were able to detect in the current study. Yet, whole blood assays' applicability may be restricted to the personalized medicine approach given its intrinsic inter- and intra-individual variability (Wouters et al., 2002). It is also important to stress out that murine cells possess danger-sensing mechanisms and maturation processes are highly similar to those of humans (Shortman and Liu, 2002) and our observation that these cells efficiently stratify the air samples according to their burden support that the murine origin of the cells is not an obstacle to the elaboration of a surrogate measure centered on human health.

5. Conclusions

This study unravels that modification of the rate and magnitude of dendritic cell activation could mitigate the immunogenic impact of complex bioaerosols, and it thus contributes to further understand the inadequation between endotoxin levels and the pathogenicity of the bioaerosols found in heavily contaminated environments. Our study also identifies murine BMDC cultures as potential biosensors with the ability to rank samples of air according to their burden and thus support their utility for the dissection of the immunopathogenic mechanisms of complex air samples.

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Authors' contributions

All of the authors contributed to the design of the study, revised the manuscript and approved its final version. MB performed all experimental procedures, contributed to the analysis of the data and to the drafting of the manuscript, which was done in collaboration with PBL, CD and DM. CD, MV, VL and PBL overlooked the validation of the air sampling protocol as well as the characterization of the samples. DM supervised the study, the in vitro experiments and the analysis of the data.

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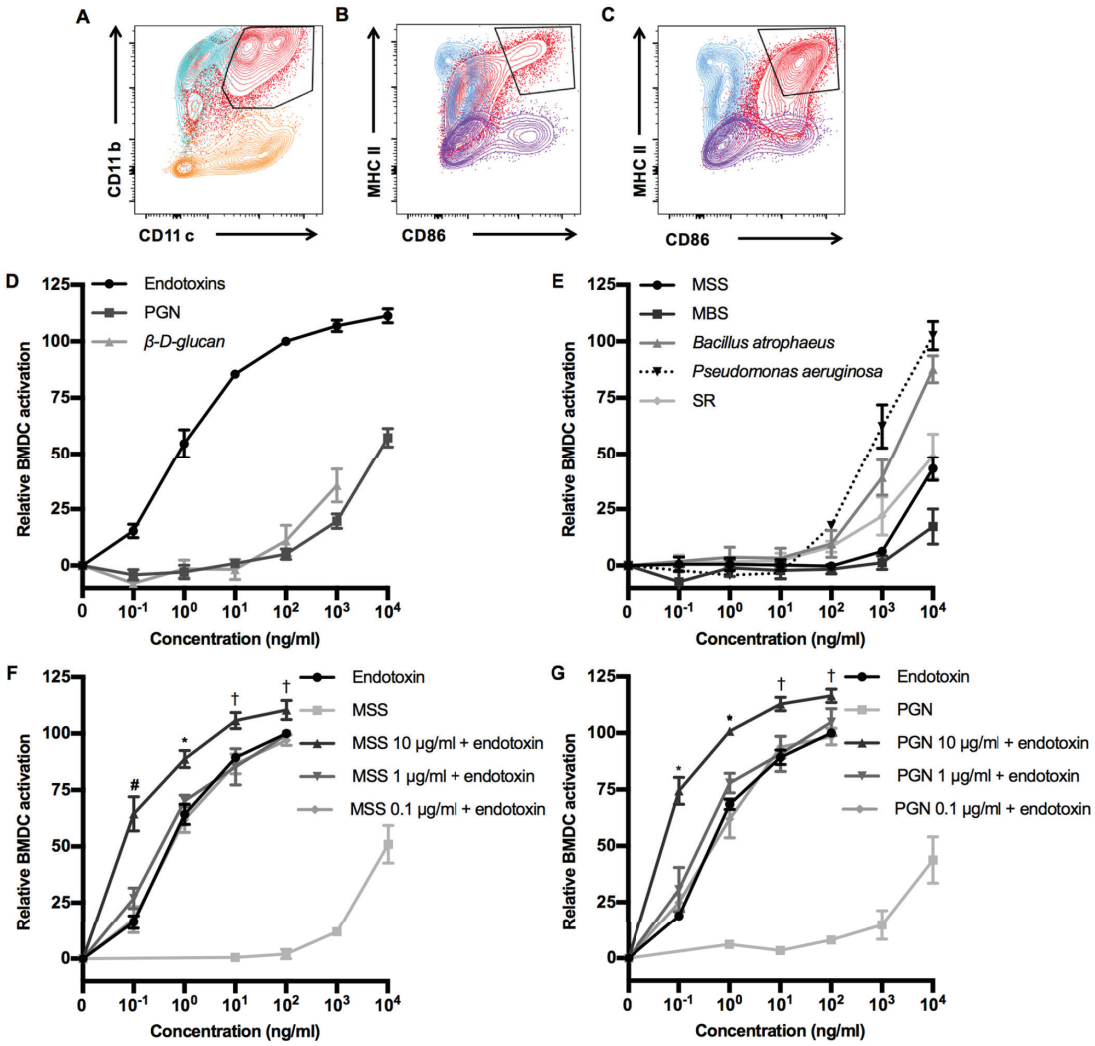

Fig. 1. The differential BMDC activation by individual or combined bioaerosol components. (A) The whole BMDC pool was initially defined as the $CD11c^{+}$ $CD11b^{+}$ cell population. The red contour lines represent a sample incubated with endotoxins (0.1 ng/ml). Turquoise and orange contour lines represent fluorescence minus one (FMO) controls. (B-C) From the $CD11c^{+}$ $CD11b^{+}$ population, MHC II^{hi} $CD86^{hi}$ cells were selected as an index of activation. Red contour lines show cells incubated with (B) 0.1 and (C) 100 ng/ml endotoxins. Blue contour lines and purple contour lines represent FMO controls. (D) BMDCs were incubated with increasing concentrations of endotoxins (n = 11), peptidoglycans (n = 8) or β-D glucans (n = 3) and (E) with MSS (n = 10), MBS (n = 7), *Bacillus atrophaeus* (n = 5), *Pseudomonas aeruginosa* (n = 3) or SR (n = 3) for 24 h. Indicated concentrations of *(F)* MSS, or *(G)* PGN were added in conjunction with with 0.1 ng/ml to 100 ng/ml of endotoxins. On the x axis, "0" was added to represent samples incubated with the vehicle. Shown is the relative BMDC activation expressed as a percentage of the 100 ng/ml endotoxin condition. Values are expressed as the mean ± SEM. $p < 0.05$. * Different from matched concentrations of endotoxins alone and MSS or PGN alone. # Different from matched concentration of endotoxins alone. † Different from matched concentrations of MSS alone or PGN alone. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

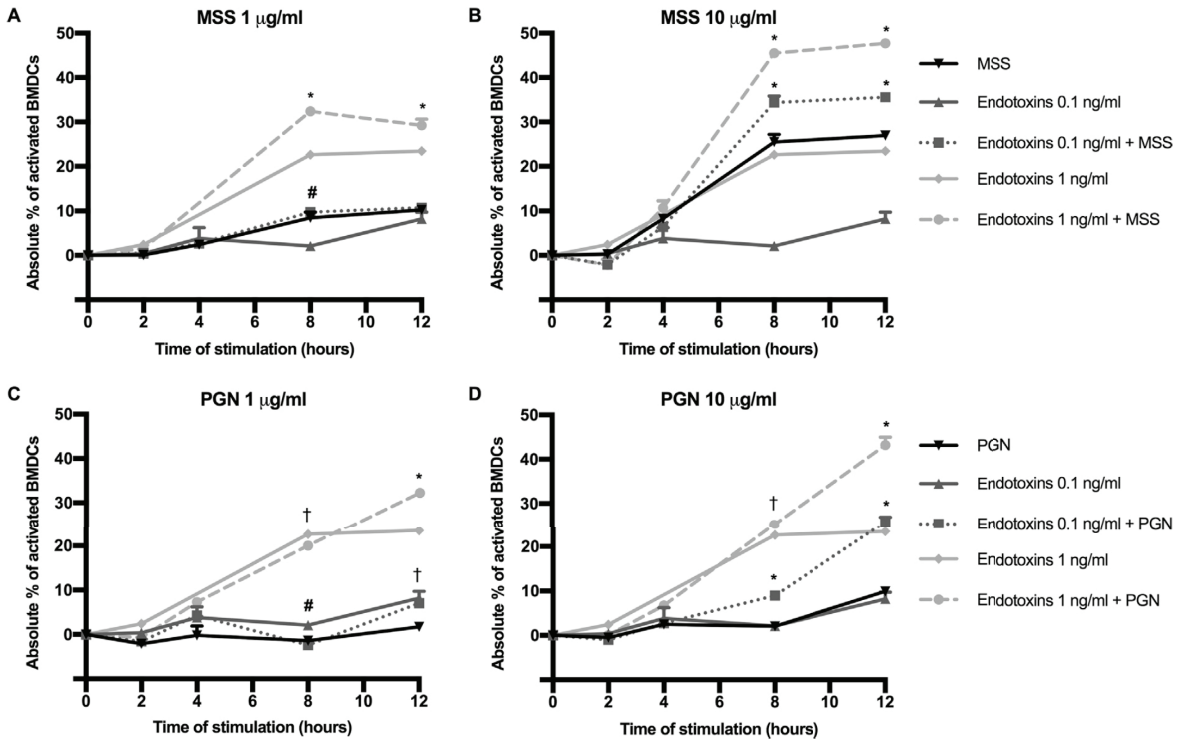

Fig. 2. Combination of agents modulates the kinetics and the magnitude of BMDC activation. BMDCs were indubated with 100; 1; 0.1 ng/ml of endotoxins and with (A) 1 μg/ml or (B) 10 μg/ml of MSS; or (C) 1 μg/ml or (D) 10 μg/ml of PGN and harvested at different time points. Absolute frequency of activated BMDCs is shown. Experiments were made in triplicate. Shown is one of two independent experiments obtained with the same results. Values are expressed as the mean ± SEM. *shows significant differences of combined agents compared to endotoxin alone and MSS alone or PGN alone. #shows significant differences of combined agents compared to their paired concentration of endotoxins. †shows significant differences of combined agents compared to MSS alone or PGN alone. $p < 0.05$.

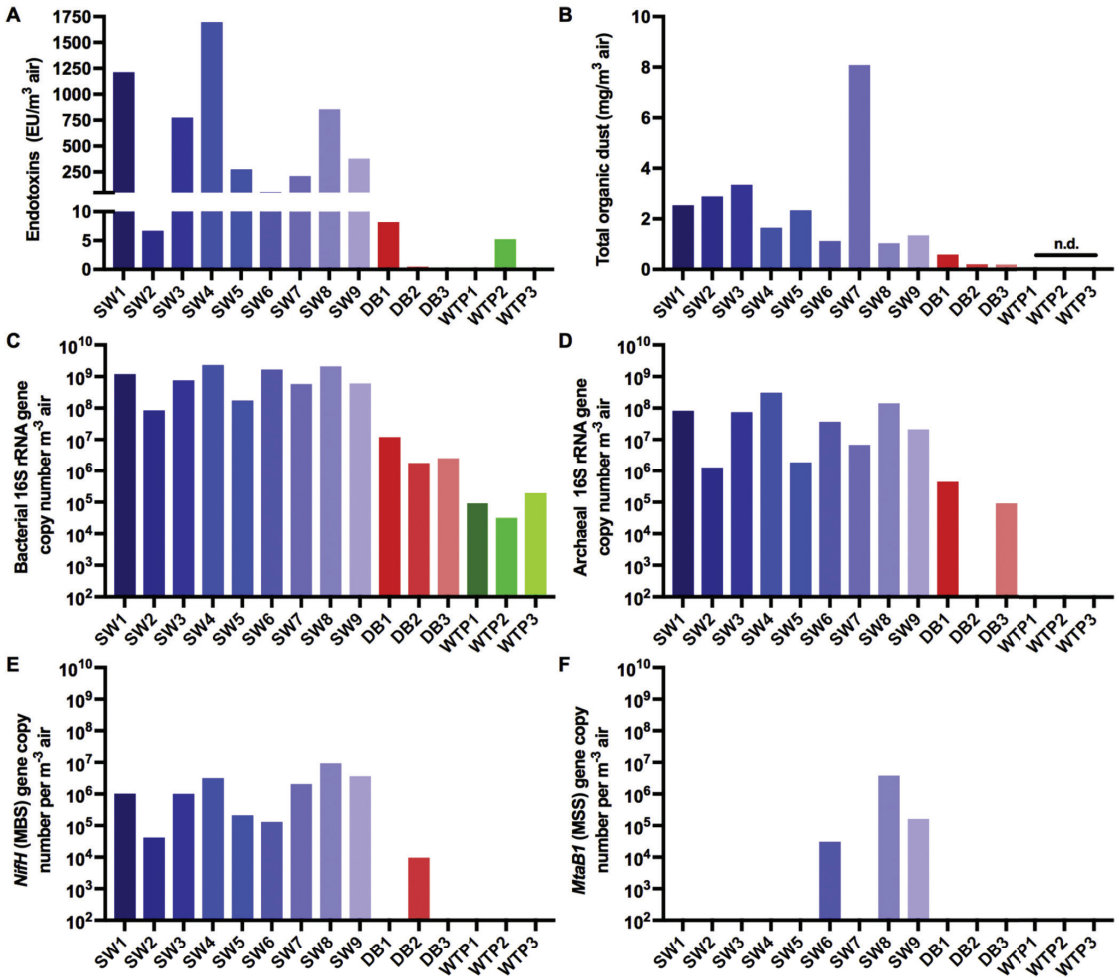

Fig. 3. Characterization of environmental samples. (A) Endotoxins. (B) Total organic dust. (C-D) Bacterial and archaeal 16S rRNA gene counts. (E) Airborne MBS *NifH* gene count. (F) Airborne MSS *MtaB1* gene count. n.d.: not determined.

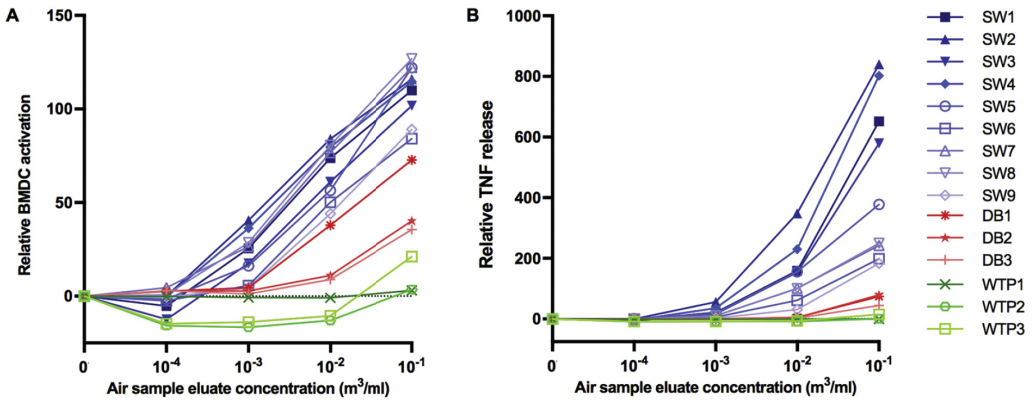

Fig. 4. Differential BMDC activation by environmental samples. BMDCs were incubated for 24 h with vehicle (represented with "0" on the x axis), or with increasing concentrations of environmental air sample eluates (ASE), corresponding to the indicated volume of sampled air per ml of culture medium. (A) The relative BMDC activation and (B) relative TNF release were recorded and expressed as the percentage of the BMDC activation induced by 100 ng/ml of endotoxins. Swine barns (SW), Dairy barns (DB), wastewater treatment plants (WTP). Experiments were made in duplicate.

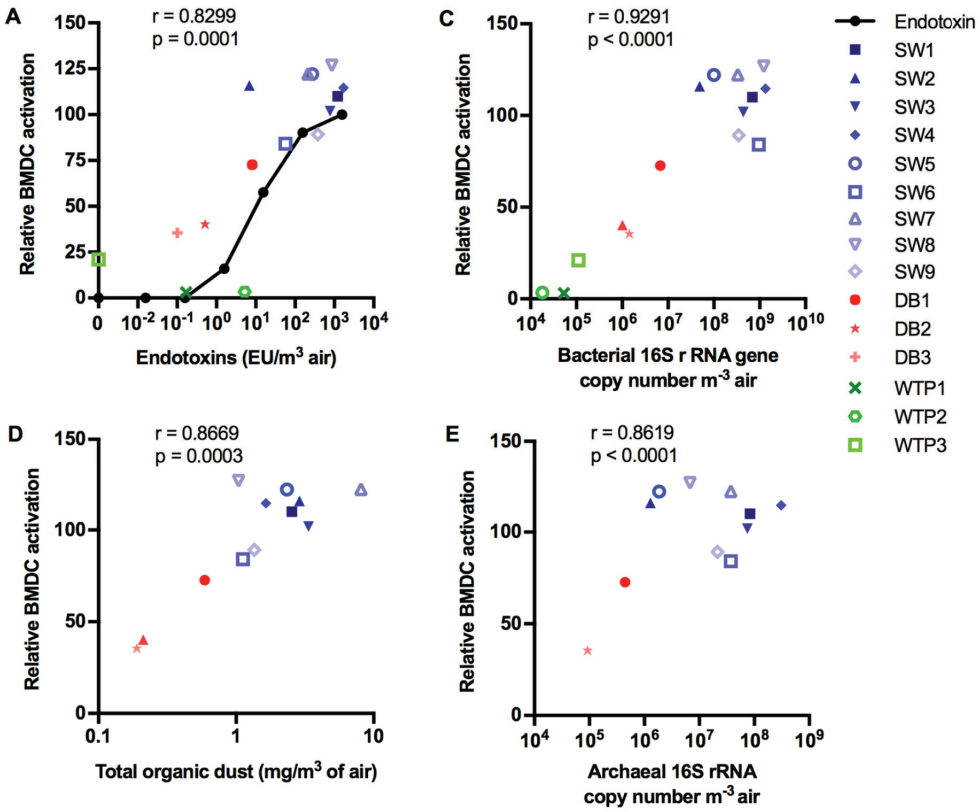

Fig. 5. BMDC activation correlates with the amount of total organic dust, endotoxins and total bacteria in airborne samples. (A) The relationship between endotoxin content in sampled environments and relative BMDC activation is shown. The number "0" was added to the x axis to denote samples were endotoxins were not detected. The black curve represents the purified endotoxins standard curve. Correlations between BMDC activation (0.1 m^3 of ASE) and (B) total bacteria or (C) total organic dust or (D) total archaea were also assessed. Relative BMDC activation is expressed as the percentage of the group incubated with 100 ng/ml of endotoxins. The correlation coefficient (r) and *p* values are shown for experimental samples in each individual panel.