Paradigms and perspectives

Environmental endotoxin exposure and asthma

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Environmental endotoxin is associated with a variety of respiratory diseases that arise in occupational, agricultural, and domestic settings. Endotoxin refers to LPS or lipooligosaccharide molecules arising from the outer cell wall of gram-negative bacteria (GNB) that function as microbial-associated molecular patterns interacting with pattern recognition receptors to induce inflammation leading to asthma and asthma-like syndromes.

It has long been established that workers are exposed to dangerous concentrations of endotoxin in agricultural settings, including concentrated animal feeding operations (facilities housing swine, poultry, and other livestock), grain handling facilities, and seed and bulb processing facilities, as well as during row crop harvesting and tasks such as grain bin cleanout.[1] Workers in occupations such as wastewater treatment, cotton textile processing, industrial-scale composting, and metals machining are also at risk. The geometric mean airborne endotoxin concentrations from a variety of environments that were measured in our research projects are shown in Fig 1, *A*. Household endotoxin loads in reservoir dust (per m^2 of area sampled) are provided in Fig 1, *B*. The high endotoxin exposures experienced in the environments shown in Fig 1, *A* induce adverse respiratory effects, including nonallergic asthma, asthma-like syndrome, organic dust toxic syndrome, mucous membrane irritation, and chronic bronchitis.

ENDOTOXIN MECHANISM OF INFLAMMATION

Endotoxin in the environment exists as aggregates of hundreds of thousands of LPS molecules or as fragments of bacterial membranes or membrane blebs. To activate signal transduction leading to inflammation, these aggregates and membrane-bound endotoxin molecules need to be extracted and presented to Toll-like receptor 4 (TLR-4). Four cell surface and free extracellular host proteins are a requisite for maximum responsiveness to endotoxin. They are LPS binding protein (LBP), cluster determinant-14 (CD-14), lymphocyte antigen 96 (MD-2), and TLR-4.[2] These host proteins interact sequentially with LBP, catalyzing the transfer of endotoxin molecules from aggregates or GNB membranes to soluble or membrane-bound CD-14. Complexes of single endotoxin molecules and CD-14 then combine with extracellular MD-2 or with MD-2 associated with membrane-bound TLR-4. Simultaneous engagement of MD-2 with endotoxin and TLR-4 results in cell activation and initiates intracellular signaling that releases proinflammatory mediators and recruits neutrophils to the lung. Murine endotoxin inhalation studies demonstrate a dose-dependent increase in TNF-α, macrophage inflammatory protein-1α, IL-6, granulocyte colony-stimulating factor, KC, and IL-1β; peribronchiolar neutrophilia; release of lactate dehydrogenase; and increased levels of markers of oxidative stress.[2] In Fig 2, it is shown that these outcomes do not occur in TLR-4 mutant or CD-14 or MD-2 knockout mice. If monomeric LOS complexed with MD-2 is administered to $MD\text{-}2^{-/-}$ mice, the inflammatory pathway is restored. The portion of the endotoxin molecule that triggers TLR-4 signaling is the phylogenetically conserved lipid A moiety that is embedded in the GNB cell outer membrane. Lipid A normally consists of 6 acyl chains attached to 2 linked glucosamine molecules. However, there are GNB endotoxins that contain only 5 acyl chains, and these have been shown in murine pulmonary exposure studies to induce minimal inflammation.[2]

Human inhalation exposure studies to endotoxin have validated findings from epidemiology and animal studies on the action of endotoxin as an innate immune-activating agent.[3] Analysis of induced sputum from human subjects exposed to 20,000 endotoxin activity units (EU) demonstrated increased neutrophils, eosinophils, IL-6, and TNF-α in both normal subjects and those with atopic asthma who were assessed 6 hours after exposure. Cell surface expression of CD-14 also increased significantly in both groups of subjects, whereas expression of TLR-4 and TLR-2 increased only in the asthma-free subjects.[3]

ENDOTOXIN EXPOSURE ASSESSMENT AND EXPOSURE CONTROL

Endotoxin exposure is commonly assessed from active air samples, passive electrostatic dust collector samples, or vacuumed reservoir dust samples. Samples are then extracted into an aqueous medium comprising pyrogen-free water (PFW), PFW with Tween 20, or TRIS-EDTA in PFW. Using [^{14}C]-3-OH-fatty acids produced from a metabolically labeled *Neisseria meningitidis* serogroup B strain and *Escherichia coli* CL99, we demonstrated that TRIS-EDTA provided the highest degree of release of endotoxin from whole GNB, GNB membrane blebs, and aggregates of endotoxin from environmental samples.[4] TRIS-EDTA was particularly effective for samples in which the endotoxin was held in bacterial membranes. Two commercial assay methods are in common use for endotoxin quantification, and both are based on the bioactivity of the hemolymph of the horseshoe crab (*Limulus polyphemus*): the kinetic chromogenic *Limulus* amebocyte lysate assay and the recombinant factor C assay. We analyzed 912 paired samples from agricultural settings by both

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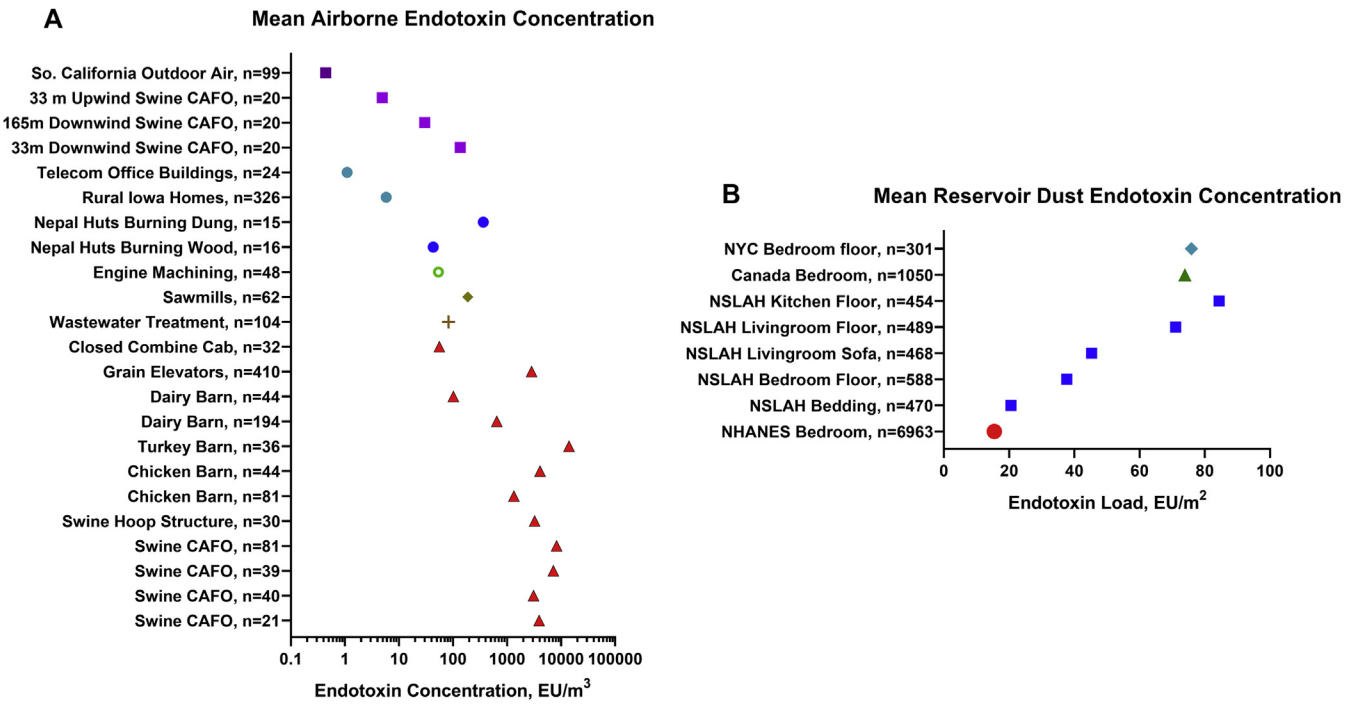


FIG 1. Geometric mean airborne endotoxin and reservoir dust endotoxin in a variety of settings. **A,** Airborne endotoxin concentration (in EU/m^3) measured in 23 environments by using the kinetic chromogenic *Limulus* amoebocyte lysate assay in our laboratory. It is noteworthy that endotoxin concentrations vary more than 10,000-fold across these locations and circumstances. Squares denote outdoor environments, circles denote indoor domestic and office locations, and red triangles denote agricultural settings from multiple studies. **B,** Reservoir dust endotoxin load (EU/m^2) measured in residences by using the kinetic chromogenic *Limulus* amoebocyte lysate assay. Squares denote data from the National Survey of Lead and Allergens in Housing (NSLAH), and the circle denotes data from NHANES. All assays were conducted in our University of Iowa Laboratory. *CAFO,* Concentrated animal feeding operation; *NYC,* New York City.

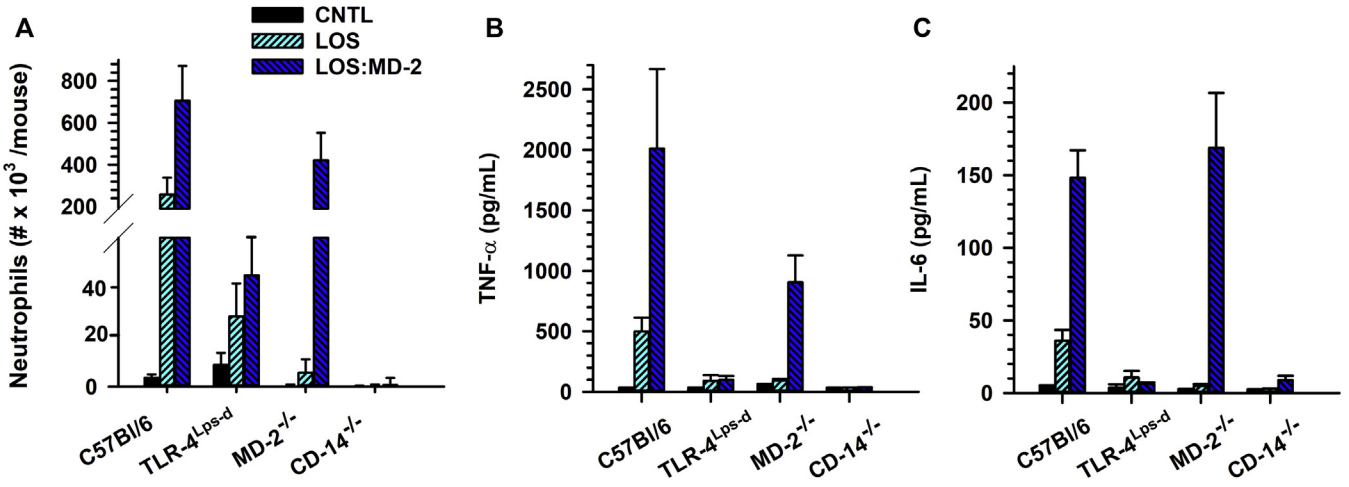


FIG 2. Four strains of mice (C57Bl/6, TLR-3^{Lps-d}, MD-2$^{-/-}$, and CD-14$^{-/-}$) were either exposed to 300 EU of lipooligosaccharide (LOS) from *Neisseria meningitidis* alone or LOS complexed with MD-2 (LOS:MD-2) or given vehicle control by intranasal instillation. The numbers of neutrophils (**A**) and cytokines (TNF-α [**B**] and IL-6, [**C**]) in their bronchoalveolar lavage fluid were measured. The mice deficient in CD-14 did not mount an inflammatory response to the LOS alone or to the monomeric LOS:MD-2 complex. Similarly, TLR-4–deficient mice had limited responses. The MD-2$^{-/-}$ mice did not respond to the LOS but did demonstrate a robust response to the LOS:MD-2, showing that exogenously administered MD-2 complexed with endotoxin could restore the pathway to inflammation.

methods and showed that they yielded comparable estimates of airborne endotoxin concentration (Pearson correlation = 0.91; $P < .0001$; slope = 0.979 ± 0.017).[5]

Endotoxin exposure control in occupational settings requires limiting exposure to tasks that generate high concentrations of inhalable bioaerosols, such as high-pressure spraying; power

washing; auguring, sifting, or mixing grain; or handling poultry and livestock, especially in confined spaces. Well-designed local and general exhaust systems and use of a powered air-purifying respirator are essential. In residential, office, and school settings, source control, high-efficiency particle arrest filtration, and frequent cleaning with high-efficiency vacuum cleaners are the most effective measures to reduce endotoxin exposure.

EPIDEMIOLOGIC STUDIES OF ENDOTOXIN

Many epidemiologic studies have assessed the role of endotoxin in asthma and wheeze. Urban cohort studies have provided clear evidence that higher levels of endotoxin in house dust are positively associated with a higher prevalence of wheeze but lower allergy.[6] Studies in children have shown that growing up on a family farm with higher exposure to endotoxin leaves a child less likely to develop allergies and allergic asthma.[7] This protective effect has received support from studies demonstrating a shift away from an allergic phenotype with early-life exposure to endotoxin. However, studies from Australia, Europe, New Zealand, and the United States found the same or higher asthma rates (ie, no protective effect) between farm and nonfarm children in 1 or more study groups.[6]

Children from 2 genetically similar agrarian founder populations were studied to assess the role of endotoxin and the environmental microbiome in innate and adaptive immunity.[7] Amish and Hutterite populations both engage in dairy farming but in different ways. The Amish apply nonmechanized, 19th century farming methods, and children enter the livestock barns from early life. Hutterites use modern farming practices, and young children have less animal exposure. Among the Amish, intense and sustained exposure to endotoxin and microbes activate innate immune pathways that shape and calibrate downstream immune responses. The result of this is a very low prevalence of allergy and asthma in Amish children that is not seen in Hutterite children.[7] Exposing mice to ovalbumin (OVA) in an asthma model along with airborne dust collected in Hutterite homes induced lung eosinophilia and bronchial hyperreactivity equal to or greater than exposure to OVA alone. However, mice exposed to Amish house dust with OVA had few eosinophils in their lung lavage fluid and blunted airway reactivity. Importantly, the Hutterite house dust collected by using an electrostatic dust collector contained 648 EU/m^2 of endotoxin, whereas the concentration of endotoxin in Amish airborne dust was nearly 7 times higher (4400 EU/m^2 [$P < .001$]).[7]

Domestic epidemiologic studies of endotoxin and asthma often rely on measuring endotoxin in vacuum-sampled reservoir dust. The geometric mean loads of endotoxin from several studies from our group, including the National Health and Nutrition Examination Survey (NHANES) and the National Survey of Lead and Allergens in Housing (NSLAH), are shown in Fig 1, *B*. In NHANES, the largest national study of endotoxin ever conducted, the concentration of endotoxin in composite reservoir dust samples collected from bedding and bedroom floors was positively associated with wheeze in the past 12 months, wheeze during exercise, doctor and/or emergency room visits for wheeze, and use of prescription medications for wheeze.[6] When associations between endotoxin exposure and outcomes were stratified by sensitization status, exercise-induced wheeze was associated primarily with nonsensitized participants, whereas wheeze and use of prescription medications for wheeze were more frequent in sensitized participants. Significant predictors of higher endotoxin exposures were lower family income; Hispanic ethnicity; participant age; dogs, cats, cockroaches, and/or smokers in the home; and having carpeted floors.[6] Coexposure to dog and cat allergens was found to increase the association of endotoxin with asthma and wheeze.[8] Further studies in this cohort showed that predictors of endotoxin exposure and the association of endotoxin with asthma and wheeze differed across climate regions in the United States.[9] In very cold and cold regions of the country, endotoxin exposure was positively associated with any wheeze, exercise-induced wheeze, prescription medication for wheeze, and doctor and/or emergency room visit for wheeze. Endotoxin exposure was positively associated with asthma and wheeze outcomes but negatively associated with sensitization to inhalant allergens in hot and humid climates. These differences could explain why studies conducted in a single city or state often yield differing results. Analysis of geocoded NHANES data along with monitored and modeled air pollution data demonstrated that coexposure to elevated concentrations of residential endotoxin and ambient $PM_{2.5}$ in adults and children and NO_2 in child participants was synergistically associated with increased visits to emergency departments for asthma.[10]

The evidence that endotoxin inhalation induces innate immune responses and lung inflammation is overwhelming.[2-4,7] However, in most circumstances airborne endotoxin exposure is accompanied by varying amounts and types of bacteria, fungal spores, and microbial-associated molecular patterns. Thus, in some circumstances endotoxin exposure assessment may represent a proxy for other elements of the environmental microbiome that display a more complex array of immune active stimuli.[6] This was evident in the study of Amish and Hutterite children in which the microbial composition of dust samples from Amish homes differed markedly from that of dust samples from Hutterite homes and led to dramatic differences in the innate immune phenotypes found between the 2 groups of children.[7] Intervention studies that target allergens and endotoxin in residences and schools also affect the concentrations and, likely, the diversity of the microbial environment in ways that may alter innate and adaptive immunity.

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