

# Pulmonary innate inflammatory responses to agricultural occupational contaminants

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**Abstract** Agricultural workers are exposed to many contaminants and suffer from respiratory and other symptoms. Dusts, gases, microbial products and pesticide residues from farms have been linked to effects on the health of agricultural workers. Growing sets of data from in vitro and in vivo models demonstrate the role of the innate immune system, especially Toll-like receptor 4 (TLR4) and TLR9, in lung inflammation induced following exposure to contaminants in agricultural environments. Interestingly, inflammation and lung function changes appear to be discordant indicating the complexity of inflammatory responses to exposures. Whereas the recent development of rodent models and exposure systems have yielded valuable data, we need new systems to examine the combined effects of multiple contaminants in

order to increase our understanding of farm-exposure-induced negative health effects.

**Keywords** Lung inflammation · Pig and chicken farms · Pesticides · Animal models · Human exposures

## Historical perspectives on occupational hazards of farming

Some of the oldest known occupational hazards are associated with farming. As early as 1555, Olaus Magnus wrote that grain dust could damage vital organs of thrashers in his book titled, “*Historia de gentibus septentrionalibus*” (Schenker et al. 1998). Subsequently, Bernardino Ramazzini documented the risks of inhaling grain dust in his work “*De Morbis Artificum Diatriba*” (Schenker et al. 1998). Despite the recognition of the respiratory hazards of farm work as early as 1555, only during the last few decades systematic investigations into the respiratory health of agriculture workers have been started. This has led to the development of field of agricultural medicine within the broader domain of occupational medicine. The biomedical data collected so far counter the myth that agricultural environments in rural settings are inherently safer than urban environments.

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## Development of concentrated animal feeding operations and negative impact on barn worker’s health

The model of animal production operations started to change in the 20th century mainly to alter and enhance food production systems. These changes were largely attributable to a need to increase food production to keep up with the rapid

increase in the world population. The increase in the world population was the result of reduced child mortality and increased life expectancy because of advances in medical care. The availability of methods to produce fertilizers and new genomics methods led to significant increases in agriculture productivity. To improve the utilization of the feed and the land used for animal-based food production, intensive animal production systems such as concentrated animal feeding operations (CAFOs) were developed (Casey et al. 2015). These CAFOs achieve efficiency in production but generate a variety of occupational contaminants that negatively impact barn workers, nearby communities and the environment (for a review, see Charavaryamath and Singh 2006). The majority of the contaminants are of animal origin (manure, urine and gas) with contributions from other sources such as feed, pesticides and biological/chemical feed additives. Among the animal origin contaminants, microbes or microbial products are at the center because of their abundance and health impact. The barn environment contains many bacteria, bacterial DNA, lipopolysaccharide (LPS), peptidoglycan (PGN), fungal cell wall components and viral particles (Corzo et al. 2013). Among the 150 or more gaseous contaminants recorded, methane (CH<sub>4</sub>), ammonia (NH<sub>3</sub>), hydrogen sulfide (H<sub>2</sub>S) and carbon dioxide (CO<sub>2</sub>) are monitored and assessed for their impact on human and animal health (for a review, see Schenker et al. 1998).

Barn workers, veterinarians and people in the vicinity of CAFOs are exposed to many microbial and chemical molecules and experience symptoms ranging from irritation of the skin, mucus membranes and eyes, runny nose, stuffy nose, cough, asthma, exacerbation of pre-existing asthma, asthma-like syndrome, bronchitis, chest tightness and excessive mucus production, changes in mood and depression (Charavaryamath and Singh 2006; Viegas et al. 2013). Studies have been carried out in which healthy human volunteers have been exposed to the swine barn environment followed by the measurement of lung function and markers of inflammation. The recent development of rat and mouse models to mimic the exposure experienced by human workers is facilitating advances in the elucidation of the molecular and cellular mechanisms of lung inflammation following exposure to barn air (Charavaryamath and Singh 2006; Senthilselvan et al. 2009; Sethi et al. 2013). Notwithstanding the advances made so far, a significant amount of work still needs to be done on the pulmonary effects of exposure to the agriculture environment.

To mitigate rising food demands, agricultural farming has witnessed a high use of pesticides. “Pesticide” is a generic name that includes insecticide, herbicide and fungicide (Mamane et al. 2015). Pesticides significantly enhance agriculture productivity (Rekha et al. 2006) and are used on about one-third of agricultural products, making their use indispensable in agricultural production (Liu et al. 2002). However, heavy use of

these chemicals results in direct or indirect toxicity and a variety of effects on non-target organisms (Ansari and Kumar 1988). Occupational exposure to various pesticides occurs mainly via dermal routes followed by inhalation and ingestion, whereas the dietary route mainly accounts for non-occupational exposure (Baldi et al. 2012). Although most pesticides are neurotoxic, respiratory dysfunctions have also been observed following direct (inhalation) or indirect (oral) exposure to pesticides in workers engaged with pesticides. The deleterious effects of pesticide exposure on target organs such as liver and kidney are well documented; however, similar data are lacking with respect to non-target organs such as lungs.

## Occupational exposure and lung inflammation

### Organic dusts

Multiple research groups have identified organic dusts as a major problem in lung inflammation on farms, particularly in CAFOs. Exposure studies in animals (Charavaryamath et al. 2005; Schneberger et al. 2016) and human subjects, evaluation of pulmonary health of workers (Senthilselvan et al. 1997a) and testing of dust mitigation strategies (Senthilselvan et al. 1997b) have shown that organic dust and its components are a primary concern in these environments. The data have also shown that all dusts are not equal. Therefore, total dust in the air may not always be directly comparable with the symptoms seen, meaning that other factors such as particle size, concentration of bacterial products (e.g., LPS and PGN), weight and composition are likely to be of great importance to the generated symptoms (Kirychuk et al. 2010; Hawley et al. 2015). The fraction of dust within the respirable range (1–10 μm) is a likely factor in deciding the inflammatory potential of barn dust and can vary depending on the type of facility (Dosman et al. 2006); however, clear effects of inhaled particles are observed outside of this range in cell cultures that differ in responses to respirable-sized particles (Hawley et al. 2015). These factors may also affect one another, such that LPS are suggested to be enriched in smaller particles (Jones et al. 1984).

Work on these particulates has focused largely on the bacterial components from these environments. Much of the research has been directed primarily towards three components of these dusts: LPS, lipoproteins and proteoglycans. Whereas these components are clearly vital to the responses seen in workers, these components are accepted not to be sufficient to explain all observations. Therefore, we will probably find more contributors to these dust-induced innate immune responses (Schneberger et al. 2016). Similarly, we might find components that are inhibitory or modulatory as opposed to stimulatory (Poole et al. 2011). As an example, organic barn dusts have recently been discovered to contain proteolytic

properties (Romberger et al. 2015) that can stimulate protease-activated receptors (PARs) 1 and 2 in the bronchial epithelium. These receptors require the cleavage of the N-terminal domain by proteases in order to induce signaling when the new terminal domain binds the receptor (Dery et al. 1998). Cooperation of PARs with other innate receptors such as Toll-like receptor 4 (TLR4) has also been suggested (Rallabhandi et al. 2008). In the PAR study, a reduction in pro-inflammatory cytokines and reduced bronchoalveolar lavage (BAL) cellular infiltrates were seen when protease activity was blocked. These proteolytic properties of organic dusts require further work, as some chemokines are produced in forms that become more potent upon such cleavage (Berahovich et al. 2005), although the study suggests that the main effect is probably through these PAR receptors.

## Gases

Barns for animals such as pigs and poultry are kept closed for biosecurity reasons and also in harsh winter climates. The closed environment leads to the build up of a variety of gases and microbial molecules. Three gases are commonly recognized as being elevated in most CAFO operations, namely ammonia (NH<sub>3</sub>), hydrogen sulfide (H<sub>2</sub>S) and carbon dioxide (CO<sub>2</sub>), although they are far from being the only ones (mentioned earlier).

NH<sub>3</sub> is highly soluble and should not penetrate far beyond the upper airway (Landahl and Herrmann 1950). It is a respiratory irritant, however and may be adsorbed to dust particulates in the air. Donham and colleagues (2002) reported synergy between ammonia and organic-dust exposure in poultry operations; this synergy explains the decline in the forced expiratory volume (FEV) and forced expiratory flow (FEF) of the lungs of workers (Donham et al. 2002). Others have reported interactions with endotoxin exposure in pigs (Gustin et al. 1994). A later study of humans with grain dust and ammonia (Sigurdarson et al. 2004) and pigs in a controlled exposure study (Done et al. 2005) failed to yield similar inflammation, whereas another suggested some effect of ammonia in response to *Mycoplasma hypopneumonia* and a clear effect of particulates on the same condition (Michiels et al. 2015). Thus, the impact of ammonia on or with dust exposures on the immune response remains uncertain.

H<sub>2</sub>S is commonly recognized as a serious potential health hazard in CAFO operations, although it is mostly associated with issues of waste management and removal. This gas can easily penetrate to the alveolar level and is often cited as a potential health hazard in these facilities, although the hazardous effects often relate to the neurological depression of respiration rather than to inflammation (Truong et al. 2006). This gas has also been shown to potentially cause cellular damage via the generation of reactive oxygen species (Eghbal et al. 2004). H<sub>2</sub>S further appears to be anti-inflammatory in several exposure

systems, although some debate remains with regards to the importance of kinetics (Whiteman et al. 2010), because most of these systems do not involve the inhalation delivery of H<sub>2</sub>S. One study did indeed suggest potential advantages to H<sub>2</sub>S in mitigating damage attributable to LPS exposure (Faller et al. 2012). Unfortunately, no work appears to exist on the co-exposure effects of this gas on organic-dust immune responses.

CO<sub>2</sub> may be elevated as high as 5000 ppm or greater in some facilities (Clark and McQuitty 1988; Ouellette et al. 1999) but is often discounted as having any effect as responses to CO<sub>2</sub> are typically mild and do not include lung irritation at levels that can be detected in such barn facilities (Langford 2005). Studies with cell cultures and animal models have yielded mixed results as to the pro- or anti-inflammatory nature of hypercapnia (Abolhassani et al. 2009; Wang et al. 2010) but have involved levels far in excess of workplace levels. A pilot project that we recently undertook to address this by using a barn-dust exposure system showed that, even at the 8-h 5000-ppm acceptable safe limit, significant changes occurred to the levels of interleukin-6 (IL-6), KC, CCL9 and MMP9 in mice (J. DeVasure, K. Bailey, D. Romberger, T. Wyatt, and D. Schneberger, unpublished data). Some of these, such as CCL9 showed clear CO<sub>2</sub> dose-specific increases at the mRNA and protein level. In almost all cases, these changes were only present upon co-exposure with barn-dust extracts, suggesting that gas exposures can modify responses to such dusts while being relatively innocuous on their own.

## Pesticides

Farm workers are more prone to pesticide exposure via dermal and inhalation routes and commonly exhibit high prevalence of cough, breathlessness, phlegm, sinusitis, throat discomfort, asthma, chronic bronchitis and allergic rhinitis (Mamane et al. 2015). Pesticide applicators using paraquat, parathion, malathion and thiocarbamate frequently suffer from reduced lung function (Mekonnen and Agonafir 2004) and evoked respiratory symptoms of wheezing (Hoppin et al. 2002). Workers processing pesticides show a high prevalence of acute respiratory symptoms during the work shift (Zuskin et al. 2008). Chronic exposure to pesticides such as chlorpyrifos, carbamate and diazinon are associated with higher incidences of lung cancer (Beane Freeman et al. 2005) and sarcoidosis (Newman et al. 2004). These epidemiological studies indicate that direct or indirect exposures to various types of pesticides adversely affect the pulmonary health of exposed workers.

The prevalence of pesticides in the food chain is responsible for short- and long-term exposures to these pesticides. Oral exposures to various classes of pesticides have been linked with pulmonary damage in various laboratory animals (Abdelsalam 1987; Karaoz et al. 2002; Yavuz et al. 2008; Merkowsky et al. 2016; Pandit et al. 2016). Organophosphate pesticides (OPs) commonly induce pulmonary edema characterized by

bronchoconstriction, congestion and intra-alveolar hemorrhage (Abdelsalam 1987). Acute OP poisoning (150 mg/kg) results in intra-parenchymal vascular congestion, thrombosis and hemorrhage, together with an increase in the proliferation of the respiratory epithelium, in the number of alveolar macrophages, in emphysematous changes and in bronchoalveolar hemorrhage in rabbits (Yavuz et al. 2008).

Similarly, chlorpyrifos, an OP, induced mononuclear cell infiltration in peribronchial and perivascular areas, hyperplasia of type II pneumocyte and increased connective tissue in the lungs of rats (Karaoz et al. 2002). In our previous study, we also demonstrated that chlorpyrifos at 3 mg/kg per day orally for 60 days causes perivascular and peribronchial mononuclear cell infiltration and septal congestion in the lungs of mice (unpublished data). Inhalation of chlorpyrifos and cypermethrin mixture leads to fibrosis with collagen proliferation, aggregation of the lymphoid cells in peribronchiolar tissue, severe dilation and congestion of the blood vessels and hyperplasia in the bronchiolar and lining epithelium of rat (Noaishi et al. 2013).

Diazinon, another OP, has predominately household usages and its exposure induces hemorrhage, infiltration of macrophages and mononuclear cells, edematous tissue, pyknotic nuclei and necrotic cells in the lungs of guinea pig (Rady 2009) and rat (Najafi et al. 2014). Fenitrothion (20 mg/kg) by oral gavage for 28 consecutive days causes the disruption of the alveolar walls and lining of the terminal bronchiole, swollen alveolar cells, infiltration of inflammatory cells and cell necrosis in rats (Budin et al. 2012). Aspiration of OP results in pulmonary neutrophil sequestration, alveolar hemorrhage and interstitial edema together with the disruption of the alveolar-capillary membrane (Hulse et al. 2014). Further, early-life exposures to OPs such as dialkylphosphate adversely affect lung function (Raanan et al. 2015). Paraquat, a herbicide, induces severe lung injury characterized by interstitial edema, leukocyte infiltration, alveolar hemorrhage, fibroblast proliferation and increased collagen deposition leading to pulmonary fibrosis (Satomi et al. 2004; Tomita et al. 2007).

We recently reported the first data concerning pulmonary inflammation in mice following oral exposure to fipronil (Merkowsky et al. 2016) and imidacloprid (Pandit et al. 2016). Imidacloprid orally at 1/20th of LD50 for 30 days resulted in lung inflammation characterized by peribronchial and perivascular infiltration and an expanded perivascular space in mice (Pandit et al. 2016). An accumulation of inflammatory cells around the terminal bronchioles, dilation of the perivascular spaces, enlargement of the airway epithelial cells and an increase in the accumulation of inflammatory cells in the alveolar septa and the alveoli were noted following both oral and intranasal exposure to fipronil (Merkowsky et al. 2016). Dopamine and amiloride-sensitive cation channels aid in lung liquid clearance and disorders of their regulation can elicit pulmonary hemorrhage and edema (Adir and

Sznajder 2003). The differential expression of dopamine receptors D2 and D4 together with amiloride-sensitive cation channel 2 resulted in pulmonary hemorrhage and edema in rats following exposure to paraquat (Satomi et al. 2004). The data suggest that oral exposures to various classes of pesticides elicit lung inflammation frequently characterized by pulmonary edema, hemorrhages and fibrosis.

Pulmonary fibrosis is another major lesion observed following exposure to various pesticides. Paraquat selectively accumulates (6 to 10 times) in the epithelial cells of the lung (Dinis-Oliveira et al. 2008) in an energy-dependent mechanism (Rose et al. 1976) suggesting that some transporter and/or ion channels are cystic fibrosis transmembrane conductance regulators (CFTR; Satomi et al. 2004) and may be involved in the onset of cystic fibrosis (Naren et al. 1999). Further, pesticide induces the altered expression of various tissue-specific genes, may be responsible for the development of pulmonary fibrosis and will be discussed later while dealing with pesticide-induced immunomodulations in the next section.

### Use of animal models to understand mechanisms of lung inflammation

The use of animal models to understand the cell and molecular mechanisms of lung inflammation is becoming an invaluable tool. Donham and Leininger housed rabbits and guinea pigs in the barn for 12 months and reported diffuse interstitial histiocytic pneumonia, epithelial hyperplasia and metaplasia with submucosal plasma cell and heterophil infiltration in tracheal and turbinate tissues and the presence of precipitins to barn-dust extract in the plasma (Donham and Leininger 1984). The authors concluded that 12 months of continuous housing in the swine barn environment induces chronic low-grade inflammation. These early studies in which laboratory animals were housed continuously in the barn failed however to model the occupational exposure of full-time barn workers.

Our group designed a rat model of human occupational exposure to swine barn air, wherein rats were exposed to the swine barn air for 8 h per day, for 1, 5 (Monday to Friday), or 20 days (for 4 working weeks, Monday to Friday). During the weekends, rats spent the full 48 h in the standard laboratory animal housing facility. During exposure, rat cages were hung at 2 m above the ground. We showed that single and multiple exposures induced lung inflammation and airway reactivity with an increase in mucus-producing cells with long-term exposure. Further, a 20-day exposure dampened airway reactivity indicating an adaptive response. Our study also recorded high amounts of LPS and viable bacteria in the barn air during the exposure periods (Charavaryamath et al. 2005). To our knowledge, this was the first known rat model of human occupational exposure to swine barn air. Our model was unique in that we mimicked human exposure of full-time barn



workers (8 h/day, Monday to Friday) and recapitulated some of the features of human occupational lung diseases with *in vivo* cell and molecular details (Charavaryamath et al. 2005). Using a similar rat model of single-day barn exposure, we demonstrated the recruitment of pulmonary intravascular monocytes/macrophages (PIMMs) at 48 h post barn exposure (Gamage et al. 2007). PIMMs are resident cells in species such as horses, cattle and pigs, whereas recruitment of PIMMs is seen in infectious and inflammatory conditions in other species (Charavaryamath et al. 2006; Schneberger et al. 2012). These cells are important since they have been shown to produce both pro- and anti-inflammatory cytokines to regulate lung inflammation (Charavaryamath et al. 2006). Depletion of PIMMs has been shown to curtail lung inflammation in various models of lung inflammation thereby underscoring their importance (Singh et al. 2004; Parbhakar et al. 2005; Gill et al. 2008). Our group has also published extensively on the biology of recruited and resident PIMMs in various species of domestic animal (for a review, see Schneberger et al. 2012). We have also shown that barn-exposed rats retain the ability to mount an effective inflammatory response to a secondary microbial challenge such as *Escherichia coli* LPS (Fig. 1; Charavaryamath et al. 2008b). Following barn exposure, we revealed the recruitment of neutrophils and monocyte/macrophages into lung tissue and lavage fluid following 1-, 5- and 20-day exposure to barn air (Charavaryamath et al. 2005, 2008a). Further, following 5- and 20-day exposure, we demonstrated the peribronchiolar and perivascular recruitment of eosinophils (Charavaryamath et al. 2005). Interestingly, eosinophil recruitment is not observed after a 1-day exposure to barn air. Following the barn exposure of rats, a secondary *Escherichia coli* LPS challenge results in a robust inflammatory response indicating the priming of lung innate immunity (Charavaryamath et al. 2008b).

These data showed that rodent models can mimic the lung inflammation observed in workers in the pig barns.

The swine barn environment is rich in bacterial endotoxin/LPS and LPS is sensed by the host pattern recognition receptor (PRR) known as TLR4. In order to delineate the role of LPS signaling via TLR4, we exposed C3HeB/FeJ mice (wild-type for *tlr4*) and C3HeB/HeJ mice (mutant for *tlr4*) to swine barn air in a similar experimental design at the same study setting. We demonstrated that swine-barn-air-induced lung inflammation but not airway reactivity, is dependent on an intact *tlr4* gene (Figs. 2, 3). We also observed *tlr4*-independent airway epithelial damage in both the strains of mice (Charavaryamath et al. 2008a). These experiments were crucial, since 10 % of the human population shows polymorphism in the *tlr4* gene and exhibits selective resistance to the effects of LPS exposure (Lorenz et al. 2001). This might explain why some newly employed workers experience severe symptoms and leave the swine production industry, whereas

those who continue to work seem to experience the “healthy worker survivor effect” (Arrighi and Hertz-Picciotto 1994).

## Occupational exposure and pulmonary immune modulations

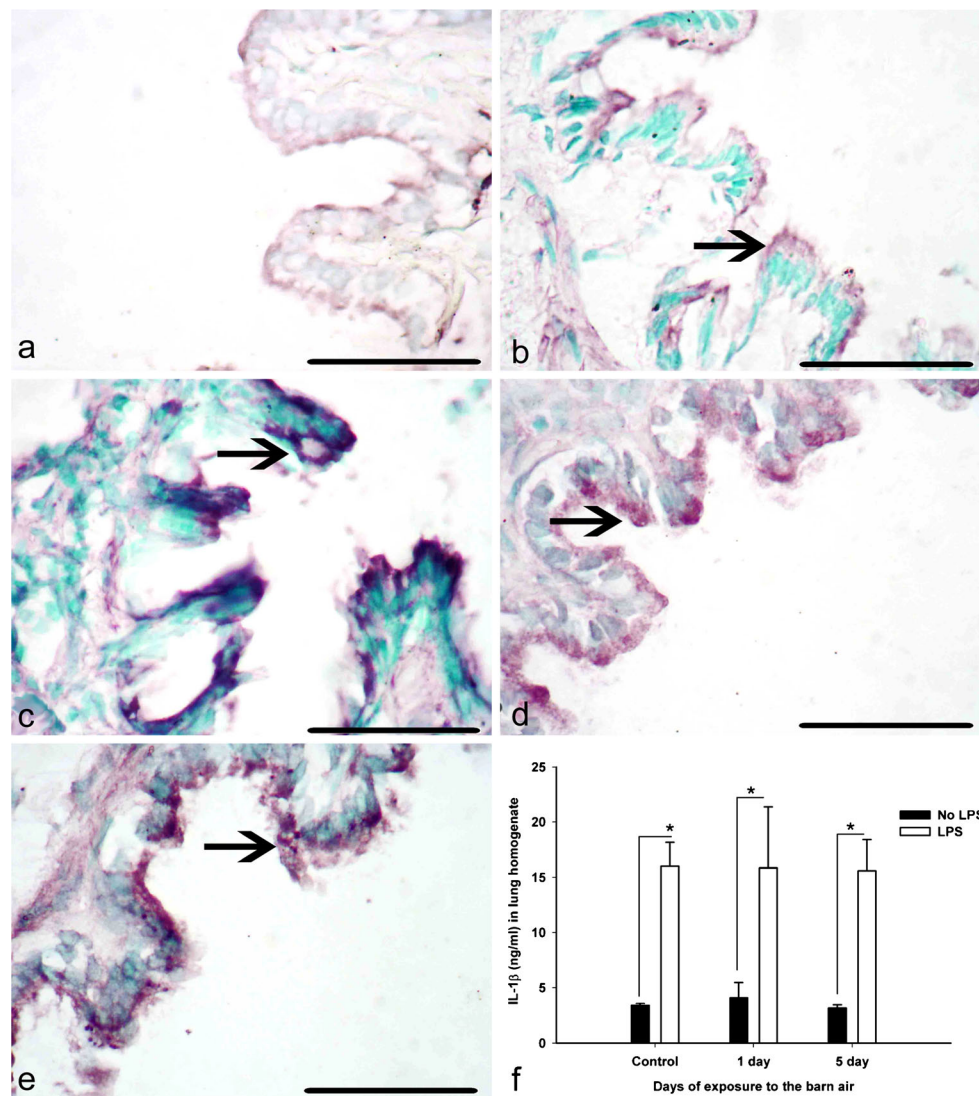
### Barn-air exposure

The importance of endotoxin and peptidoglycan in barn-dust inflammation rests in large part on the responses of the innate immune system. The innate immune system is organized to act rapidly to potential pathogens, responding to conserved microbial motifs that it recognizes as foreign and initiating immune responses. Since the discovery of Toll receptors in insects (Hashimoto et al. 1988) and their homologs in mammals and other species (Medzhitov et al. 1997), a large and diverse family of innate immune receptors have and probably will continue to be found.

Possibly the most studied innate immune receptors are in the TLR family. The number of TLRs vary between species, with ten TLRs being recognized to date in humans. These receptors can detect a broad range of lipids, proteoglycans, proteins, RNAs and DNAs (Akira and Takeda 2004). In all cases, TLRs signal through the same pathway (except for TLR3, with TLR4 signaling through the shared and alternate pathway), recruiting the MyD88 protein, which initiates a signaling cascade leading to induction of a number of immune-related pathways such as P38 and NF- $\kappa$ B (nuclear factor kappa-B; Akira and Takeda 2004).

Subsequently, additional families of innate immune receptors have been added to the TLRs. The NOD-like receptors are cytoplasmic receptors that mimic some of the binding range of the TLRs, signaling to the NF- $\kappa$ B and mitogen-activated protein (MAP) kinase pathways (Chen et al. 2009). NALP receptors constitute another 14 receptors that are part of the NOD family. Additional receptors include carbohydrate-binding C-type lectins such as collectins (discussed later) and RIG-1-like receptors (Kawai and Akira 2008), pentraxins (Du Clos 2013) and complement (Dunkelberger and Song 2010), to name just a few. Whereas many of these have yet to be explored with regard to barn dusts, some work has shown changes to complement (Von Essen et al. 1988) and NOD signaling (Poole et al. 2011). For the purposes of this review, we will focus primarily on TLRs, especially TLR2 and TLR4, in relation to organic-dust exposures.

TLR4 is perhaps the best studied of the TLRs. While it is most noted for binding to LPS (with CD14, MD-2 and LBP), the range of ligands has expanded over time to encompass other microbial products and markers of cellular or tissue stress/damage (Vaure and Liu 2014). Evidence exists for the wide expression of the receptor in the lung on the bronchial epithelium, pulmonary epithelial cells, vascular endothelium,



**Fig. 1** Immunohistochemical expression of interleukin-1 $\beta$  (IL-1 $\beta$ ) protein (arrows) in rat lung sections. Lung sections from controls (a, b) and 1-day barn-exposed rats (c, d) without and with *Escherichia coli* lipopolysaccharide (LPS) challenge, respectively and from 5-day barn-exposed rats (e) without *Escherichia coli* lipopolysaccharide (LPS)

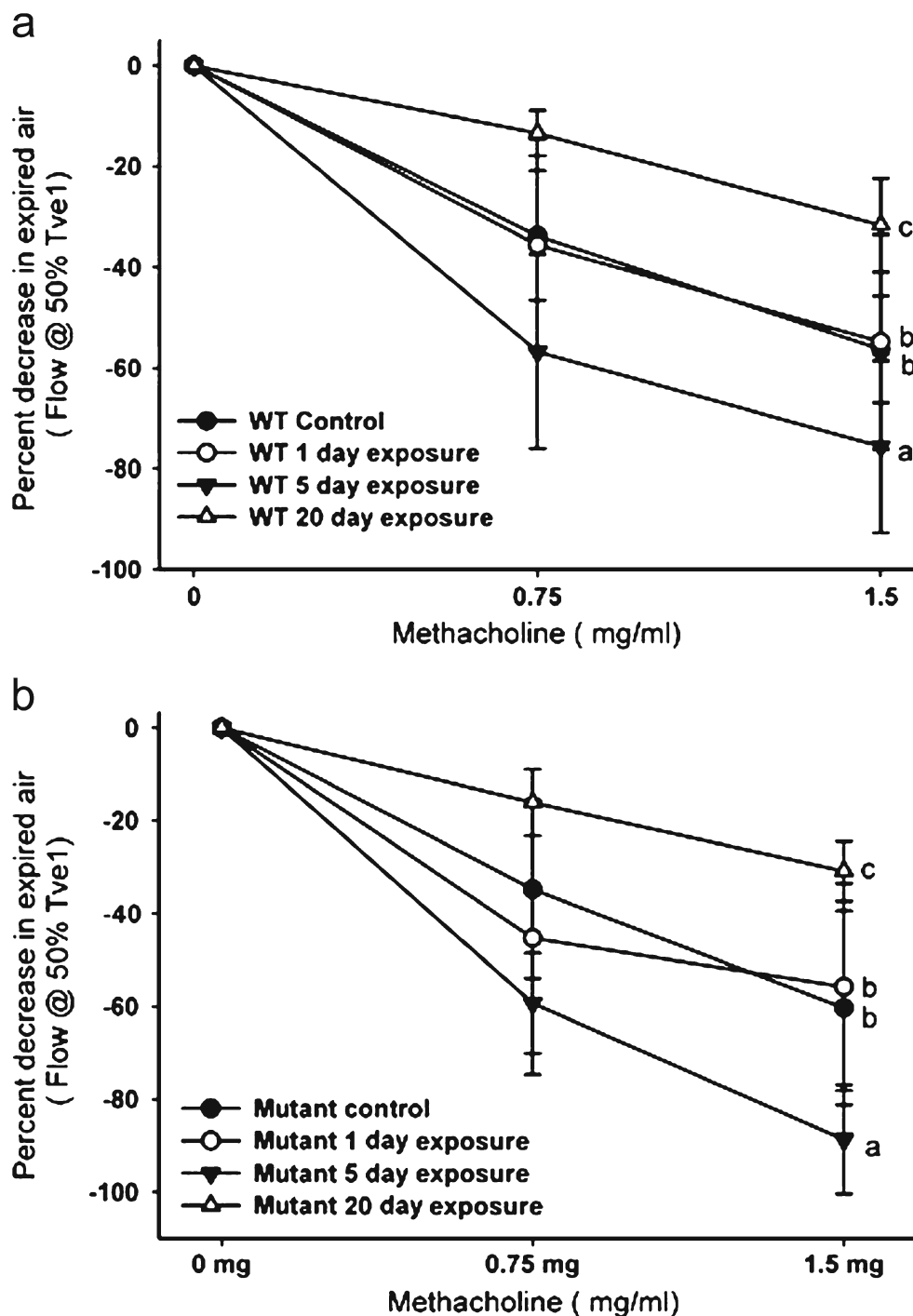
challenge (data not shown for 5-day barn-exposed rats with *E. coli* LPS challenge). **f** Quantitative representation of IL-1 $\beta$  protein concentrations from control, 1-day and 5-day barn-exposed rats without and with *E. coli* LPS challenge (\* $P < 0.001$ ). Reproduced with permission from Biomed Central (Charavaryamath et al. 2008b)

and macrophages (Janardhan et al. 2006) but activation can also be restricted by required cofactors such as MD-2, e.g., in the stimulation of airway epithelial cells (Jia et al. 2004). In humans, endotoxin exposure in swine barns has long been known to be related to respiratory health status (Vogelzang et al. 1998) and studies on human subjects have shown that the status of the TLR4 gene is associated with increased lung dysfunction after barn exposure (Senthilselvan et al. 2009), clearly establishing TLR4 and its ligand LPS as an important factor in organic-dust-induced lung inflammation. These results have been extended beyond swine barns to chicken barn dusts (Kirychuk et al. 2010) showing that these dusts enhance TLR4 expression, at least in the short term but not with long-term chronic exposure (Pallvi et al. 2016).

TLR2 is typically associated with the detection of peptidoglycans, lipoteichoic acid, lipopeptides, lipoproteins and zymosan (Zahringer et al. 2008). This TLR can dimerize with TLR1, TLR6, or TLR10, greatly increasing the range of possible ligands that it can detect (Oliveira-Nascimento et al. 2012; Kang et al. 2009; Oosting et al. 2014). In the lungs, TLR2 is expressed on type-2 epithelial cells, bronchial epithelium and alveolar macrophages (Bailey et al. 2008). Studies have shown that Gram-positive bacteria tend to predominate in the barn environment (Martin et al. 1996) suggesting that TLR2 may be even more important than TLR4 in determining the response to these organic dusts.

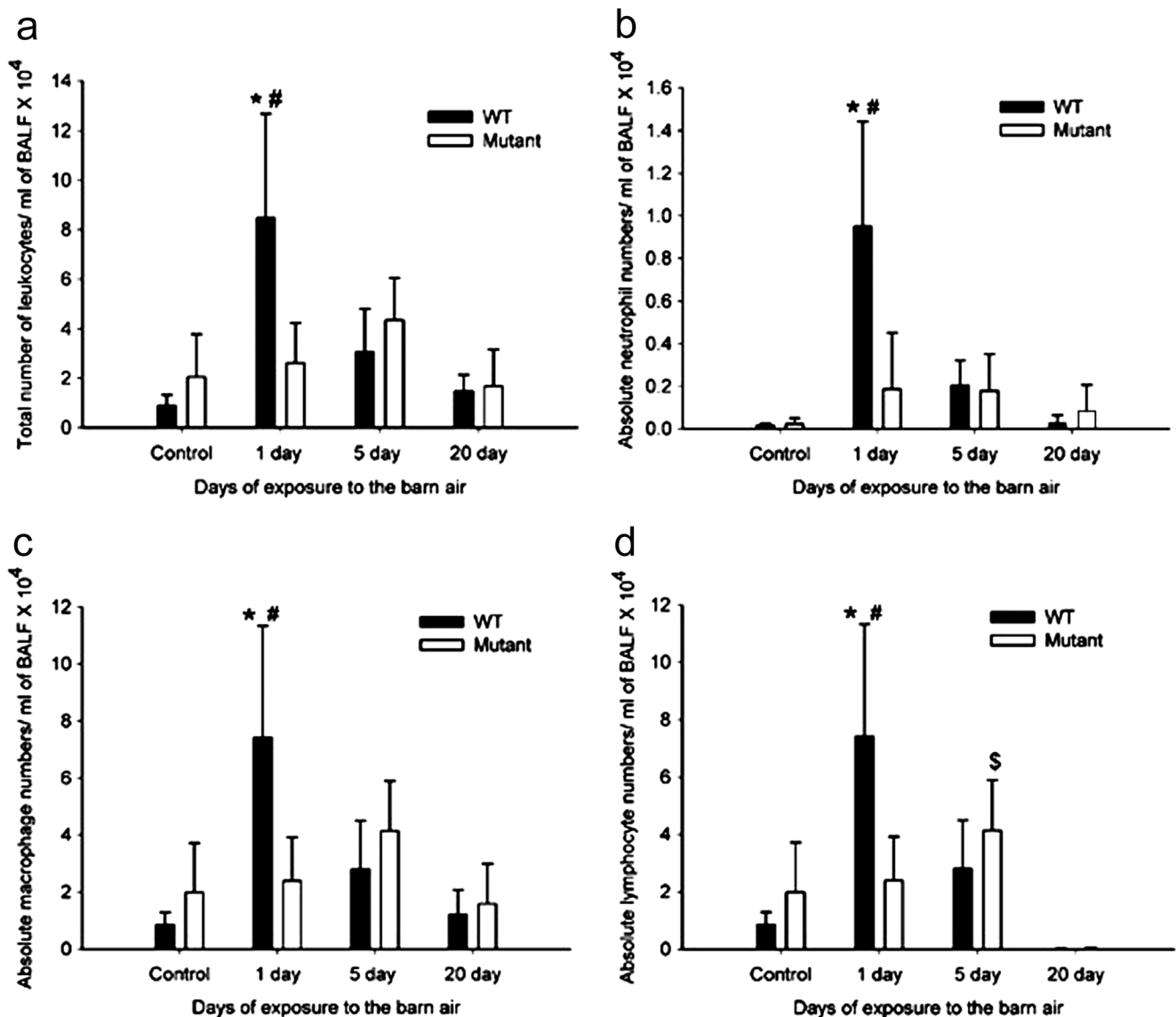
Some work has also been done with TLR9 and organic dusts (Batzer et al. 2007; Bauer et al. 2013). TLR9 detects

**Fig. 2** Airway hyper-responsiveness (AHR) to increasing doses of methacholine was similar in Toll-like receptor 4 (TLR4) wild-type (WT; **a**) and mutant (**b**) mice. After 5- and 20-day exposure, significantly increased and dampened AHR were seen, respectively. Reproduced from Charavaryamath et al. (2008a) with permission from Taylor and Francis



microbial DNA and thus would be expected to respond to any bacterial DNA in dusts. Similar to the other TLRs, widespread expression of the receptor occurs in the lung (Schneberger et al. 2013). Like TLR4 and TLR2, the expression of the receptor can also be found on dendritic cells (Hoene et al. 2006) and neutrophils (Hayashi et al. 2003). Although nowhere near as strong a response as TLR2 and TLR4, changes have been seen in BAL cellular composition in TLR9 knockout mice exposed to the barn

environment (Schneberger et al. 2016) and changes to levels of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interferon- $\gamma$  (IFN- $\gamma$ ) have been detected in the lung. Another study of these same animals revealed what appeared to be reduced numbers of type-II cells in exposed knockout animals after chronic barn-air exposure (Sethi et al. 2013). These results help to illustrate that multiple active components make up such organic dusts and that the response generated to them spans multiple



**Fig. 3** Total and differential leukocytes in bronchoalveolar lavage fluid (BALF) were counted after Wright's staining. One-day barn-exposed WT mice showed higher total leukocyte numbers (a), absolute neutrophils (b), absolute macrophages (c) and absolute lymphocytes (d) than 1-day

barn-exposed mutants and all other WT groups. Five-day barn-exposed mutants showed higher absolute lymphocyte numbers compared with 20-day exposed mutants (d). Reproduced from Charavaryamath et al. (2008a) with permission from Taylor and Francis

receptors. Whereas TLR9 may not express the magnitude of response seen with TLR2 and TLR4, it also appears to contribute to organic-dust immune responses.

Although intracellular signaling often occurs through shared pathways, the regulation and diversity of responses can also be achieved through a variety of cofactors that are required for function or that enhance or inhibit function. For example, CD14, MD-2 and LBP are required as co-factors for functional binding of TLR4 (Heumann et al. 2003). In cases of tissue or cell injury, released factors such as HMGB1 are known to signal or enhance response of TLRs 2, 4 and 9 (Park et al. 2004; Ivanov et al. 2007) in various cell types. Many TLRs also have a function in the detection of stress or

injury. For example, TLR9 can detect and respond to mitochondrial DNA and thus can also sense necrosis (Zhang et al. 2010), while TLR4 can distinguish between long- and short-chain hyaluronan (Jiang et al. 2005), the latter of which is a signal of tissue damage.

With most TLRs sharing a common pathway leading to NF- $\kappa$ B activation, interest has been shown in knocking out the adapter protein MyD88, which initiates signaling along most all TLR pathways (Kawai et al. 1999). Knockout of MyD88 is highly effective in greatly reducing lavage cell numbers in response to organic dust, in addition to a number of key cytokines and chemokines both in acute and chronic exposures. Oddly, parenchymal inflammation is greater in the



dust-exposed MyD88 knockout animals. Many of these aggregates are composed mainly of B and T cells (Bauer et al. 2013). Similarly, whereas MyD88-knockout bronchial epithelial cells are less responsive to the organic-dust-induced slowing of ciliary beat, wound closure is also inhibited, suggesting a need for TLR signaling in efficient wound repair (Poole et al. 2015). The use of bone marrow mouse chimera has revealed an interesting observation that airway hyperresponsiveness to barn dust is mediated by MyD88 in structural cells and not leukocytes (Poole et al. 2015). This occurs in spite of the secretion of pro-inflammatory cytokines in the same system being highly reliant on MyD88 expression in hematopoietic/lymphocytic cells. Further, neutrophilic influx into the lung is reliant on MyD88 expression in both cell populations showing the importance of both immune and non-immune cells in the overall lung response to organic dusts (Poole et al. 2015).

More recently, the discovery of the induction of A20 by endotoxin and organic dusts has garnered attention for being necessary for protection from the development of asthma (Schuijs et al. 2015). This ubiquitin-modifying enzyme has roles in modifying responses from a broad range of receptor pathways such as TLR, NOD, TNF, IL-17 and the T-cell complex that all lead to NF- $\kappa$ B activation, showing that the effects of exposure to organic dusts can alter a broad array of immune response mechanisms (Catrysse et al. 2014). RIG-1 signaling is also modified by A20, suggesting more than just NF- $\kappa$ B signaling is altered (Lin et al. 2006).

Although NF- $\kappa$ B activation is of great importance to responses to barn dusts, evidence has been presented of other pathways of significance in these responses. Data from the Romberger, Wyatt and Poole laboratories show the role of protein kinase C (PKC) in swine-barn-dust-induced swift responses in a bronchial epithelial cell system; the response peaks at 1–2 h and is not induced by LPS alone (Romberger et al. 2002). Two isoforms of PKC, in particular PKC- $\alpha$  and PKC- $\epsilon$ , have further been shown to be essential to TNF- $\alpha$ , IL-6 and IL-8 secretions in response to swine dusts (Romberger et al. 2002; Wyatt et al. 2010), although some variation has been observed between the different types of dusts (Wyatt et al. 2007). More recently, these groups looked at means to inhibit this PKC cytokine cascade. Using a cAMP-dependent protein kinase (PKA)-activating agent, they were able to inhibit TNF- $\alpha$  production and, as a result, the downstream PKC and cytokine/chemokine activation and production (Wyatt et al. 2010). Another such attempt to enhance PKA was performed by using agonists to the  $\beta$ -adrenergic receptor, such as salbutamol and salmeterol, which show similar results (Romberger et al. 2016). In another attempt at limiting PKC activity, dimethylarginine dimethylaminohydrolase (DDAH) was found to be able to inhibit PKC and cytokine response to swine dusts, suggesting further levels of control to intracellular signaling to these dusts

(Bailey et al. 2014). These data were the first to show the role of PKC in barn-dust-induced cellular responses in bronchial epithelial cells.

### Pesticide exposure

Pesticide exposures have been associated with marked immune dysregulation and adverse cytokine profiles (Duramad and Holland 2011) suggesting that the immune system is a target of the toxic effect of several pesticides. Cytokines are the important markers revealing the nature of the immune disturbances induced by pesticide immunotoxicity (Rowsey and Gordon 1999). Increased levels of circulating cytokines and chemokines and neutrophil sequestration in the lungs are characteristics of systemic inflammation (Abraham 2003).

OPs have been involved in the manipulation of cytokines expression (Alluwaimi and Hussein 2007) and OP-induced asthma is characterized by elevated Th2 cytokines such as IL-4, IL-5 and IL-13 (Duramad et al. 2006). Parathion, chlorpyrifos and diazinon increase the mRNA expression of TNF- $\alpha$ , IL-1 $\beta$ , platelet derived growth factor (PDGF) and transforming growth factor- $\beta$  (TGF- $\beta$ ) in a concentration-dependent manner without affecting viability in a human monocyte cell line differentiated into macrophage-like cells (THP1; Proskocil et al. 2014). IL-6 and IL-13 play an important role in oxidative stress (Rangasamy et al. 2005) and their mRNA expression is up-regulated together with antioxidant defense system enzymes (Mt1, Mt2, Hmox1, Gcl, GR, Txn1) after 6 h of paraquat exposure in lungs of mice (Tomita et al. 2007).

Paraquat upregulates various cytokine, chemokines and growth factors in lungs of mice (Ishida et al. 2006; Qian et al. 2015) and rats (Tomita et al. 2002). A significant increase occurs in the gene expression of TNF- $\alpha$  and monocyte chemo-attractant protein-1 (MCP-1), macrophage inflammatory protein (MIP)-1 $\alpha$  and MIP-2, fibrogenic growth factors such as TGF- $\beta$ , platelet-derived growth factor (PDGF)-A, acidic fibroblast growth factor and hepatocyte growth factor in mice (Ishida et al. 2006). Further, paraquat-induced lung injury is characterized by significantly increased ( $P < 0.01$ ) serum TGF- $\beta$ 1, IL-10 and TNF- $\alpha$  in rats (Jiang et al. 2015) and IL-1, IL-6, TNF- $\alpha$  and total protein levels in BAL fluid in mice (Qian et al. 2015). Paraquat exposure induces the differential pulmonary expression of neurotransmitter receptor genes, transporter genes, voltage-gated ion channel genes, lipid metabolism enzyme genes, G-protein involved in endocytosis and exocytosis genes and apoptosis-related genes in rats (Satomi et al. 2004). The synergistic effects of these molecules are presumed to be the cause of pulmonary fibrosis following paraquat exposure (Ishida et al. 2006).

Pesticides may cause toxic effects by stimulating various pro-inflammatory cells such as alveolar macrophages (Banks and Lein 2012). The alveolar macrophages during asthma

secrete various pro-inflammatory mediators (Barnes 1994) that cause airway hyper-responsiveness (Zhang et al. 2007). Parathion activates alveolar macrophages to release TNF- $\alpha$ , which is linked to dysfunction of neuronal M2 receptor and airway hyper-reactivity in guinea pig (Proskocil et al. 2013). The role of alveolar macrophages is further underscored by the finding that their depletion by clodronate abrogates parathion-induced airway hyper-reactivity in guinea pig (Proskocil et al. 2013). The exposure to paraquat increases Fas and FasL expression together with CASP3 to suggest the involvement of apoptosis in lung injury (Tomita et al. 2007).

We recently reported that TLRs such as TLR4 and TLR9 together with pro-inflammatory mediators may play a role in the pulmonary effects in mice exposed to fipronil (Merkowsky et al. 2016), ethion (unpublished data) and imidacloprid (Pandit et al. 2016). TLR9, a membrane-bound receptor, is expressed on a variety of cells including macrophages and neutrophils and its capacity to bind non-methylated CpG DNA sequences (Schneberger et al. 2016) makes it central to the recognition and phagocytosis of bacteria (Murad and Clay 2009). TLR9 activates the innate immune system in response to microbial/endogenous DNA such as mitochondrial DNA (mtDNA; Zhang et al. 2010) and its expression correlates positively with the severity of lung damage (Qian et al. 2015). During pesticide-induced cellular damage and necrosis in the lung, the damaged mitochondria may leak into the extracellular space thereby triggering the release of mitochondrial-damage-associated molecular patterns (DAMPs) such as mtDNA (West et al. 2011). Hence, paraquat-induced acute lung injury with elevated pulmonary TLR9 mRNA expression might be attributable to the activation of TLR9 by mtDNA (Qian et al. 2015).

Chlorpyrifos can exhibit airway hyper-reactivity without altering acetyl cholinesterase (AChE) and M3 muscarinic receptor function on airway smooth muscle (Fryer et al. 2004) suggesting a uniqueness in the onset of airway hyper-reactivity following exposure to OPs. However, a lack of similar data exists for other classes of pesticides, especially newly evolved pesticides. Thus, the exploration of this field will be of interest in order to unfold the cellular mechanism of pulmonary immune-modulations following exposure to a specific pesticide.

### Role of surfactants

Surfactant proteins (SP) are important constituents of pulmonary defense and are a lipoprotein complex of phospholipids and apoproteins (Pastva et al. 2007). Immune surfactants A (SP-A) and D (SP-D) are now seen as critical components of innate immunity not only for the direct neutralization of pathogens and allergens but also for the inhibition or enhancement of inflammation depending on the circumstance

(Kishore et al. 2006). Generated in the lung and often in other locations, immune surfactants serve to bind and inhibit pathogens, whereas non-immune surfactants B and C have roles in the reduction of lung surface tension (Weaver and Conkright 2001). The immune surfactants or collectins, on the other hand, also carry out a diverse array of innate defense roles. They can bind a number of carbohydrate motifs, affecting direct killing and the neutralization of a broad array of pathogens (Kishore et al. 2006). Apart from this, these surfactants are known to bind the SIRP- $\alpha$  cell receptor and inhibit P38 activation under normal conditions. However, changes in SP-A or D structure as a result of binding pathogens or nitric oxide s-nitrosylation of SP-D (Guo et al. 2008) alter this binding and so these surfactants instead bind CD91/calreticulin and activate the very same P38 pathway (Gardai et al. 2003). This last action initiates a number of cellular changes that may influence innate immunity, including the alteration of TLR2, TLR4 and TLR9 expression (An et al. 2002). SP-A has further been shown to block LPS binding to CD14 (Sano et al. 1999) and LPS binding to protein (Stamme et al. 2002) and subsequently inhibit TLR4 responses, revealing yet another way in which these molecules can modify innate immunity. Conversely, non-immune surfactant protein C can enhance LPS binding to CD14 (Augusto et al. 2003) suggesting that even the “non-immune” surfactants play roles in lung innate immunity.

Pulmonary SPs are also involved in the pathophysiology of lung injury following exposure to pesticides (Gil et al. 2007). Phospholipid transfer protein is frequently expressed in lungs (Jiang and Bruce 1995) and is an important regulator of surfactant biology (Pownall et al. 1991). Pulmonary SP-A and SP-D mediate host-defense functions (Wright 2005) and are potent endogenous inhibitors of lipid peroxidation and oxidative cellular injury (Bridges et al. 2000). SP-D reduces apoptosis in alveolar macrophages to prevent abnormal alveolar remodeling during lung injury (Clark et al. 2002). Lung damage following paraquat poisoning is secondary to derangement of the pulmonary surfactant system (Fisher et al. 1975). An increase occurs in the SP-D concentration in the BAL fluid of rat following exposure to paraquat (Gil et al. 2007). However, paraquat exposure decreases the mRNA expression of SP-D in the lung of mice (Tomita et al. 2007). The data reflect the species-specific role of SPs during pesticide-induced lung damage; this needs to be explored in depth.

Although surfactants appear not to have been studied in terms of organic dust, preliminary work in our laboratory has shown SP-A and SP-D reductions in A549 lung cell lines exposed to hog barn dusts. Further studies of SP-D have suggested that excess SP-D can inhibit the production of cytokines such as IL-8 in response to these dusts (unpublished observations). This further suggests that some of the inflammatory response generated in lungs to these dusts might be attributable to reductions in these immune surfactants.

However, given their potential multiple roles in inflammation, much more work needs to be done with regard to such exposures.

### Pesticide and endotoxin interaction

Endotoxin/LPS is a major pathogenic element that can induce systemic inflammation during Gram-negative bacterial infection, as it is an integral cell wall component of these bacteria (Sugiyama et al. 2008). LPS/endotoxin has been reported to induce lung damage in mice (Takeda and Akira 2004; Pandit et al. 2016) and rats (Shang et al. 2009). LPS challenge increases the production of IL-1 $\beta$ /IL-18 (Eltom et al. 2014) resulting in pulmonary inflammation (Sugiyama et al. 2008). LPS-induced TLR4 activation increases the levels of IL-1 $\beta$ /IL-18 in the BAL fluid via an ICE protease-activating factor (IPAF)-dependent and caspase-independent pathway (Eltom et al. 2014) and of IL-1 $\beta$  and TNF $\alpha$  in the airways and epithelial cells via the NF- $\kappa$ B pathway (Takeda and Akira 2004). Further, monocytes have integral activated caspase-1 that releases active IL-1 $\beta$  after a single stimulation with LPS (Netea et al. 2009).

Pesticides and bacterial interactions mimic the over-expression of certain genes/cytokines resulting in host tissues injury (Duramad et al. 2006; Gagnaire et al. 2007). Workers, especially those employed in agriculture, animal-confinement buildings and grain-handling facilities, are exposed to higher levels of endotoxins (Burch et al. 2010). Endotoxin is typically associated with the negative respiratory symptoms in poultry workers (Just et al. 2009) suggesting that endotoxin inhalation is responsible for pulmonary dysfunction such as acute airway obstruction, hypersensitivity pneumonitis, chronic bronchitis and decreased lung function in humans (Thorn 2001). LPS exposure together with fipronil alters the expression of cytokines in human monocytic cells (Sidiropoulou et al. 2011). Similarly, chlorpyrifos and its metabolites do not induce cytokine expression *in vitro*, whereas in combination with LPS, they stimulate the expression of higher levels of IFN- $\gamma$  than LPS alone (Duramad et al. 2006).

In another study, the treatment of pregnant mothers with methamidophos and the intranasal challenge with respiratory syncytial virus (RSV) to the offspring mice suppressed the levels of IL-6 and IFN- $\gamma$  in the BAL fluid of mice (Watanabe et al. 2013). Further, methamidophos treatment did not alter the pulmonary viral titers but moderately suppressed lung inflammation and the gene expression of the cytokines in the lungs of the offspring mice. The study led to the conclusion that the exposure of the mothers to methamidophos during pregnancy and nursing resulted in an irregular pulmonary immune response in the offspring mice.

We have previously demonstrated that LPS co-exposure with imidacloprid (Pandit et al. 2016), fipronil (Merkowsky

et al. 2016) and chlorpyrifos (unpublished data) increases vulnerability to pulmonary damage. Treatment with imidacloprid orally at 1/20th of LD<sub>50</sub> for 90 days in combination with LPS leads to marked lung damage together with a significant increase in total cell and neutrophil counts in BAL and peripheral blood without altering the mRNA expression of TLR-4 and TNF- $\alpha$  (Pandit et al. 2016). The limited data available on pesticide-endotoxin interaction suggest that this aspect is a potential area of pulmonary research in the future.

### Resolution of inflammation

An exciting new area of research is the elucidation of the mechanisms involved in the resolution of inflammation. These anti-inflammatory and pro-inflammation-resolving lipid mediators such as lipoxins, resolvins and protectins promote the reduction of pro-inflammatory processes (Bannenberg 2010). This does not necessarily mean a reduction in lung immunity, as several factors are shown to increase resistance to pathogens (Chiang et al. 2012). Whereas little has been done regarding organic dusts, recent work with maresin has shown that it can inhibit the organic-dust-induced expression of IL-6, IL-8 and TNF- $\alpha$  (Nordgren et al. 2013). Animal studies have confirmed a reduction in BAL neutrophil infiltration, IL-6 and TNF- $\alpha$ , although characteristic dust-induced lymphoid aggregates remain (Nordgren et al. 2015). With such work exposures often being chronic, this suggests a new area of potential interest.

### Concluding remarks and future directions

Whereas research over the years has elucidated multiple components within dust binding to a wide variety of innate immune receptors, we still have no clear definition of all the components responsible for responses seen to organic barn dusts. Size fractionation of dusts has revealed immune activity in multiple fractions. Although some components such as LPS and proteoglycans have emerged as major components, they do not define all of the effects seen. Work by several groups has also suggested the contribution of gases in these barn workplace environments modifying responses to dusts. To date, very little work has been carried out in this area, despite several gases having long been determined as irritants. Initially, some of the more common gases will need to be tested in isolation, moving to combinations that may approximate poor ventilation scenarios. Although additional single stimulatory factors may yet still emerge, future study models will need to look at the combined effects of multiple potential workplace stimulants such as dust, pesticides and gases. Whether this is addressed through more worksite exposure models or laboratory models, the complicated nature of the

problem will eventually require more complex exposure systems to be addressed. The farming workplace environment is an intricate one and whereas we have learned a great deal since the first reports of hazards in 1555, much remains to be done if we are to preserve the health of those that provide food for us all.

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