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Selective Modulation of the Pulmonary Innate Immune Response Does Not Change Lung Microbiota in Healthy Mice

To the Editor:

Although long considered sterile, healthy lungs are now known to harbor diverse and dynamic low-abundance bacterial communities. Recent studies in humans (1) and animals (2) have revealed that lung immunity, even in health, is variable across individuals and correlated with variation in lung microbiota. Yet the causal relationships driving this correlation between lung microbiota and lung immunity remain undetermined. Does variation in lung microbiota propel variation in lung immunity activation? Or does variation in lung immunity create an altered respiratory microenvironment, resulting in altered lung bacterial communities?

A recent report in this journal by Wu and colleagues (3) demonstrated that direct modulation of murine lung microbiota results in rapid and persistent changes in lung immunity, conveying sustained protection from subsequent respiratory infection. These results reveal that the correlation between lung microbiota and lung immunity is, at least in part, attributable to the microbiome's

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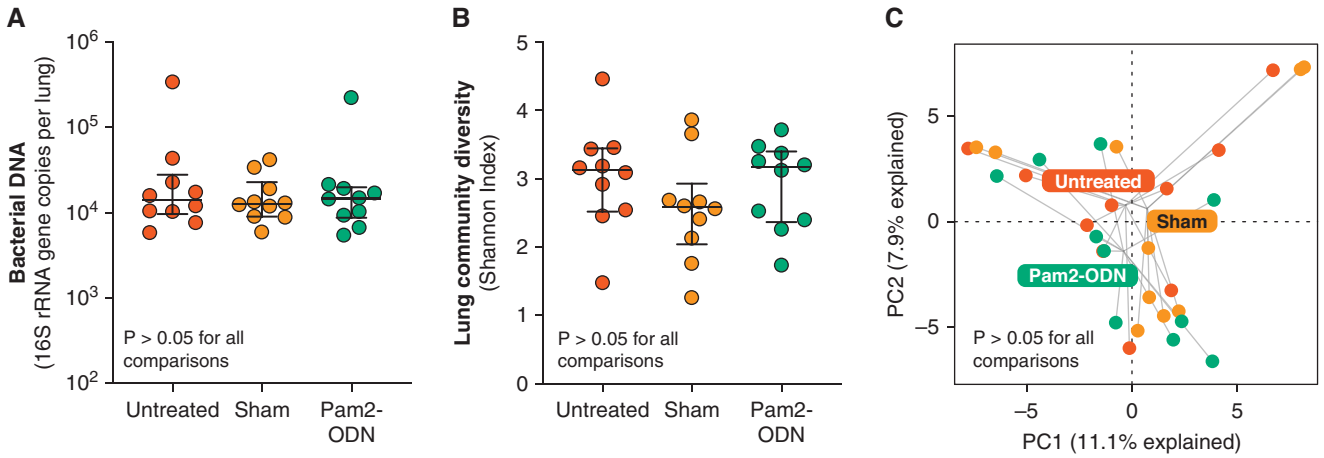

Figure 1. Experimental modulation of lung innate immune tone does not influence lung microbial communities. Healthy, adult mice received either no exposure ("untreated"), PBS inhalation ("sham"), or synergistic TLR2/6 and TLR9 stimulation via inhaled Pam2-ODN. (*A–C*) TLR agonism did not influence the total bacterial burden in murine lungs (*A*), lung bacterial diversity (*B*), or lung community composition (*C*). Horizontal lines with error bars represent median and interquartile range. Significance was determined using Kruskal-Wallis one-way ANOVA (*A*), ANOVA with Holm-Sidak multiple comparisons test (*B*), and PERMANOVA (*C*). PBS = phosphate-buffered saline; PC = principal component; PERMANOVA = permutational multivariate ANOVA; TLR = Toll-like receptor.

influence on lung immunity. Yet, to our knowledge, the inverse hypothesis has not been tested: do changes in the immune tone of healthy lungs provoke changes in lung microbiota?

We therefore designed an experiment to test the hypothesis that direct modulation of the lungs' innate immune tone causes changes in the burden, diversity, and community composition of lung bacteria. To accomplish this, we experimentally modulated the lung immunity of healthy 8- to 9-week-old adult mice (C57BL/6) using a single exposure to an inhaled agent (Pam2-ODN) that synergistically agonizes multiple Toll-like receptors (TLR2, TLR6, and TLR9). This inhaled exposure has potent effects on respiratory epithelial cell function (4) that provide robust protection against bacterial and viral respiratory infections (4, 5). Mice received either no exposure ("untreated"), phosphate-buffered saline inhalation ("sham"), or Pam2-ODN treatment (10 mice per experimental group). To avoid confounding by cohousing (2), we obtained all mice from a common breeding colony, and mice from multiple cages were randomly assigned to each experimental group, then randomized to postexposure cages. Mice were harvested 6 days after exposure. Bacterial communities in the lungs, tongue, and ceca were quantified using droplet digital PCR and identified using 16S rRNA gene amplicon sequencing as previously described (2). Lung microbiota were characterized using homogenized whole lung tissue (6). We obtained 21,850 ± 22,976 16S reads per specimen; no specimens or taxa were excluded from analysis.

Given the vulnerability of low-biomass microbiome studies to procedural and sequencing contamination (7), we first compared the bacterial taxa detected in lung specimens with those of procedural controls (Pam2-ODN, sham vehicle) and sequencing controls (blank wells, DNA extraction controls, buffers and reagents used in library preparation). Bacterial communities in lung specimens were distinct from those detected in procedural controls ($P < 0.0001$, permutational multivariate ANOVA [PERMANOVA]) and sequencing controls ($P < 0.0001$, PERMANOVA), with minimal overlap in prominent taxa. Bacterial communities in lung specimens were also distinct from those of tongue and cecal specimens ($P < 0.0001$ for both, PERMANOVA). Murine lung microbiota did not differ by pretreatment or post-treatment cage ($P > 0.05$ for both, PERMANOVA). We thus concluded that these lungs contained a bacterial signal distinct from procedural, sequencing, and anatomic control specimens. Direct comparisons of taxa in lung specimens and controls are available at https://doi.org/10.6084/m9.figshare.14347088.v1.

Modulation of lung innate immunity by one-time synergistic agonism of TLR2, TLR6, and TLR9 did not have an observed effect on lung bacterial communities (Figure 1). Compared with untreated and sham-exposed mice, Pam2-ODN–treated mice did not differ in their lung bacterial burden, diversity, or community composition ($P > 0.05$ for all comparisons, Kruskal-Wallis one-way ANOVA and PERMANOVA). No specific taxa were enriched or depleted in Pam2-ODN–treated mice relative to control groups ($P > 0.05$ via *mvabund*).

We thus concluded that in healthy mice, modulation of lung innate immune tone via one-time synergistic TLR agonism does not have a persistent effect on lung bacterial communities. Although it is possible our exposure had a transient effect on lung bacteria, by 6 days after exposure the lung communities were indistinguishable from those of control animals. Our findings complement those of a recent report by Wu and colleagues (3), in which direct modulation of the lung microbiome resulted in immediate and persistent changes in lung immunity. Of note, Wu and colleagues found no difference in lung bacterial communities in STAT3 mutant mice, which are characterized by IL-17 mediated inflammation. Taken together, these findings suggest that the established correlation between lung microbiota and lung immunity (1, 2) is more likely attributable to the

host response to respiratory microbiota rather than the microbiome being altered by variation in lung immunity.

This interpretation is compatible with our evolving understanding of the microbial ecology of the lung microbiome in health: while lung bacteria are viable (8) and metabolically active (9), they are low in abundance and highly transient (9, 10), defined by the dynamic balance between immigration (via microaspiration) and elimination (via mucociliary clearance and host defenses) (9) rather than the selective growth of resident communities (as in the lower gut). This ecologic model suggests that variation in the microbiota in healthy lungs is more likely attributable to variation in microbial immigration factors (i.e., the identity of pharyngeal microbiota and the frequency of microaspiration) than to variation in baseline lung immunity and environmental growth conditions.

We emphasize that both our findings and those of Wu and colleagues were derived from the study of healthy lungs and should not be assumed to apply to conditions of airway, alveolar, or interstitial injury. The pulmonary microenvironment is radically altered by acute and chronic disease, and diseased lungs harbor distinct microbial communities that, when compared with health-associated microbiota, are *1*) greater in abundance, *2*) less transient (e.g., the clinically familiar phenomenon of bacterial colonization), and *3*) likely reflect a more "bidirectional" relationship between the microbiome and the host immune response (11). Although we detected no appreciable effect of Pam2-ODN on lung bacterial communities 6 days after exposure, it is entirely possible that TLR agonism has a short-lived effect on lung microbiota that was resolved by the time of harvest due to the continuous exposure of the lungs to environmental microbiota (2). In addition, although our experimental exposure (Pam2-ODN) has potent and well-established effects on lung innate immunity (4, 5), it remains possible that other exposures (such as TLR or macrophage inhibition or recurrent TLR agonism) would have measurable effects on lung microbial communities. The lungs' innate immune apparatus, including TLRs, serves a "gatekeeping" role to prevent invasion and overgrowth by pathogens and proinflammatory microbiota and may thus predictably play little role in shaping lung microbiota in the absence of such microbes. TLR expression may thus play a more important ecologic role in conditions of altered respiratory microbiota (i.e., disease or dysbiosis).

In summary, we report that augmentation of lung innate immune tone via synergistic TLR agonism has no appreciable effect on the lung microbiome of healthy adult mice. Taken together with recent reports, these results suggest that the established correlation between lung microbiota and lung immunity in health reflects dynamic calibration of the host response to the respiratory microbiome. ■

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