Immunity
Letter

NLRP6 and Dysbiosis: Avoiding the Luring Attraction of Over-Simplification

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Identification of the individual factors contributing to the formation of "healthy" or disease-modulating "dysbiotic" microbiome communities is an important aspect of microbiome research. In light of the high individuality of human and animal microbiomes across geography and time, the reproducibility of findings between different labs is increasingly recognized as a challenge to this exponentially expanding field.

The NLRP6 inflammasome was one the first host factors suggested to regulate the composition and function of the intestinal microbiome. Early cross-sectional studies of wild-type (WT) and gene-deficient mice suggested that a deficiency in the NLRP6 inflammasome was associated with a state of dysbiosis, leading to aggravation of intestinal inflammation and metabolic dysfunction (Elinav et al., 2011; Henao-Mejia et al., 2012). These findings, of significant conceptual importance, encouraged further studies to explore the nature and degree of the contribution of NLRP6-deficiency versus husbandry effects on gut microbiome community structure.

The first step to assess the contribution of NLRP6 to the regulation of the microbiota, while controlling for husbandry effects, included experimentation under littermate-controlled conditions. Indeed, two labs reported that such F1 to F1 (i.e., +/− x +/−) breeding led to the development of distinct microbiome compositions in NLRP6 inflammasome-deficient mice housed in two different vivaria (Levy et al., 2017b; Seregin et al., 2017). In contrast, a recent publication by Wullaert and colleagues (Mamantopoulos et al., 2017) and another by Robertson and colleagues (Lemire et al., 2017) did not observe such genotype-dependent effects on microbiome composition. Could these conflicting results be explained by differences in microbiome compositions between different facilities?

To illustrate this possibility, one may envision a hypothetical simplified scenario. Assuming a mouse deficient in a bona-fide microbiome-modulating factor is mono-inoculated with a single commensal, such a hypothetical animal would not develop dysbiosis, as the microbial spectrum of both case and control does not allow the host factor under examination to exert its effect. In a more complex scenario, an enhanced SPF barrier facility may likewise vary in the abundance of organisms capable of mediating dysbiosis. In such case, the inability to detect differences in the microbiota between littermate WT and gene-deficient mice would simply reflect the absence of dysbiosis-triggering microbes in a given housing environment, thereby leading to over-simplified conclusions. Indeed, in the case of NLRP6, marked compositional microbiome differences existed between involved vivaria (Figure S1A, including reanalyzed data from Mamantopoulos et al., 2017).

In light of this inter-facility microbiome variability, littermate approaches must be coupled to a second important step involving generation of germ-free NLRP6-deficient and wild-type (WT) mice (or alternatively mice re-derived into enhanced specific-pathogen-free conditions), followed by spontaneous or fecal microbiome transplantation-induced microbiome reconstitution in these mice under identical conditions (Figure S1B). Using these approaches, if an identical microbiome composition develops in reconstituted genetically-altered and WT recipient mice, a genetic contribution of the host factor in question to community structure becomes unlikely. If, however, *de novo* dysbiosis does develop in reconstituted germ-free mice, then the opposite conclusion may be reached, namely of a host-derived effect on microbiome community structure that becomes apparent only in specific microbiome contexts.

Indeed, in the case of NLRP6, spontaneous recolonization of germ-free NLRP6-deficient mice yielded de-novo dysbiosis as compared to simultaneously recolonizing germ-free WT mice (Levy et al., 2015). In addition, three independent labs (spanning 7,000 km and three different continents) have recently reported on studies using the fecal transfer strategy, i.e., diverse microbiomes of different origins were concomitantly transferred into germ-free NLRP6-deficient and WT recipient mice or into WT and NLRP6-deficient mice harboring a standardized microbiota (Gálvez et al., 2017; Levy et al., 2017b; Seregin et al., 2017). Importantly, all three labs independently reported the post-transfer *de novo* development of dysbiosis in mice lacking NLRP6. Galvez et al. further showed that, while dysbiosis was not observed in the context of a low-diversity microbiome, it reemerged upon colonization with a complex microbial community, indicating that the impact of NLRP6 on microbiome structure requires the presence of certain taxonomic elements. Furthermore, two of the studies (Levy et al., 2015, Seregin et al., 2017) exogenously replenished systemic IL-18 levels in NLPR6-deficient mice, which led to reversal of NLRP6 deficiency-associated dysbiosis. Additionally, a fourth recent study (Radulovic et al., 2017) identified apigenin as an NLRP6 regulator, thereby enabling to demonstrate NLRP6-dependent microbiome modulation even in WT

mice, further ruling out confounding husbandry-related effects. Collectively, these results support the notion that NLRP6 contributes to regulation of microbiome community structure upon exposure to a sufficiently diversified microbiome. Moreover, as reported both by Flavell and colleagues (Elinav et al., 2011), other laboratories (Levy et al., 2015; Seregin et al., 2017) and by Wullaert and colleagues themselves (Mamantopoulos et al., 2017), colitis susceptibility was noted only when NLRP6 deficiency induced dysbiosis, while susceptibility was absent when dysbiosis was not present, thereby supporting the notion that the effect of NLRP6 on regulating susceptibility to intestinal inflammation is mainly mediated through its microbiome-modulatory activity.

We hope that the collective knowledge gained by the above studies will increase the awareness of the importance in rigorously integrating multiple complementary experimental modalities into microbiome research. These should include both littermate breeding strategies and fecal microbiome transplantations of diverse sources, but never solely rely on only one or the other (Figure S1B). In generalizing beyond NLRP6, utilization of such a comprehensive stepwise approach may enable a conclusive verification or falsification of the microbiome modulatory roles suggested for other innate immune receptors, including NLPR3, NOD2, and TLR5. To the best of our knowledge, contributions by these receptors were thus far disputed only by littermate experimentation (such as in Robertson et al., 2013), but not with the necessary complementary fecal transfer strategies outlined above. As in the case of NLRP6, only such an integrative approach will enable correct interpretations of host effects on microbiome community structure, while avoiding partial and non-generalizable conclusions.

SUPPLEMENTAL INFORMATION

Supplemental Information includes one figure and can be found with this article online at https://doi.org/10.1016/j.immuni.2018.04.002.

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