Chapter 14

Analysis of Microbiota Alterations in Inflammasome-Deficient Mice

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Abstract

Inflammasomes have emerged as central regulators of intestinal infection, immunity, and inflammation. Inflammasome activity mediates intestinal epithelial integrity, antimicrobial responses, and initiates inflammation through generation of the cytokines interleukin (IL-)1 and IL-18. Recent studies have identified an additional layer of inflammasome function in the intestine, namely, the control of intestinal microflora composition. Inflammasome-deficient mice show an aberrant microbial community which is dominantly transmissible to healthy mice. This dysbiosis in inflammasome-deficient mice has a profound impact on their physiology and pathophysiology, both locally in the intestine and systemically. Therefore, it is essential to consider the influence of the composition of microbial communities on experiments performed with inflammasome-deficient and other innate molecule-deficient mice, and to conduct experiments to control for potential dominant effects of the microflora on host responses. In this chapter, we provide experimental procedures to monitor inflammasome-mediated modifications of the intestinal microflora composition in mice and to test the resultant functional consequences of these changes in microbial communities and their transmission to cohoused mice.

Key words Inflammasome, Microflora, Dysbiosis, Intestine, Cohousing, 16S RNA sequencing

1 Introduction

The formation of a multi-molecular complex consisting of an upstream NLR protein, the adaptor molecule ASC, and caspase-1—named inflammasome—was first described by Juerg Tschopp and colleagues 10 years ago [1]. Since then, many biochemical and cellular features of inflammasomes have been revealed: they have been associated in several physiological and pathophysiological processes, and their mechanisms of action and triggering stimuli are under intense investigation [2]. However, we are just beginning to understand the tissue and cell type-specific roles that inflammasome complexes play in vivo. As sensors of both microbe-associated

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Christine M. De Nardo and Eicke Latz (eds.), *The Inflammasome: Methods and Protocols*, Methods in Molecular Biology, vol. 1040, DOI 10.1007/978-1-62703-523-1_14,

molecular patterns and endogenous signals of damage [3], one would predict that inflammasomes exert critical functions in organ systems which are potential entry sites for infectious pathogens, and are exposed to both microbial colonization and mechanical stress. Indeed, recent studies have identified important functions for inflammasomes at mucosal surfaces, ranging from pivotal functions in tissue homeostasis to host defense to infection [4]. Importantly, inflammasomes are involved in regulating the composition of intestinal microbial communities, and inflammasome-deficient mice feature an aberrant microbiota [5], with several specific bacterial taxa being overrepresented as compared to the "regular" microflora of a wild-type mouse (although the latter may vary significantly among animal facilities, housing conditions and diet). This aberrant microbiota triggers an enhanced inflammatory reaction in the intestine and predisposes the host to inflammatory bowel disease [5]. Moreover, inflammasome-deficient mice feature compromised intestinal barrier function, and microbial components are more prone to access the circulation, thereby also affecting distal organ systems, most prominently the liver. There, the presence of microbial products causes a basal inflammatory state, which predisposes the host to manifestations of the metabolic syndrome, such as insulin resistance and hepatic steatosis [6].

Notably, this aberrant microbial community in inflammasome-deficient mice is dominantly transferred to wild-type mice housed in the same cage, and causes the same disease manifestations in inflammasome-competent recipient mice [5]. For instance, when cohoused with inflammasome-deficient mice, wild-type mice become more susceptible to the development of nonalcoholic fatty liver disease, and show a similarly enhanced penetration of microbial products into the circulation [6]. This shows that inflammasome deficiency may not be the direct cause of disease susceptibility, but rather initiates intestinal dysbiosis, which then predisposes the host to disease. Therefore, it is extremely important to consider the impact of dysbiosis on any experimental outcome when studying the effects of inflammasome deficiencies in vivo.

Inflammasomes are not the only sensor molecules of the innate immune system which have been recognized for their role in shaping the composition of the intestinal microbiota. For instance, aberrant microbial compositions and properties have been reported for mice lacking TLR5 and MyD88 [7], indicating that the sensing of microbes, which are in contact with intestinal epithelial cells or have penetrated into the lamina propria, is crucial to maintaining a mutualistic relationship between the host and its microbial environment. Furthermore, the loss of this sensing ability results in outgrowth and abnormal behavior of bacteria that would be defined as commensals in healthy hosts [8].

Given the increasing recognition of the importance of the intestinal microbiota for both local and systemic host physiology in health and disease, it is essential that certain experimental

procedures be followed to analyze the contribution of the intestinal microbial composition to disease phenotypes of inflammasome-deficient mice. We describe here methods which are useful to monitor the intestinal microbial composition in inflammasome-deficient mice, and to test the functional impact of dysbiosis on physiology and pathophysiology. We focus on three major procedures: (1) transfer of the microflora by cohousing, cross-fostering, and oral gavage, (2) sampling and processing of fecal material, and (3) DNA sequencing and analysis methods.

2 Materials

2.1 Mice

1. C57BL/6.
2. ASC$^{-/-}$, Caspase-1$^{-/-}$.
3. IL-18$^{-/-}$, IL-1β$^{-/-}$.
4. NLRP3$^{-/-}$, NLRP6$^{-/-}$.

2.2 Consumables

1. 2 mL screw-cap tube (Axygen).
2. 1.5 mL tubes.
3. 0.1 mm glass beads, density 2.5 g/cc (BioSpec).
4. Phase-lock gel (PLG) tube (5′).
5. Plastic pestle.
6. 40 μm cell strainer (BD).
7. Gavage needle.
8. Sterile forceps.

2.3 Instruments

1. Mini-bead beater (BioSpec).
2. qPCR cycler.
3. Water bath or heat block at 50 °C.

2.4 Reagents

1. TNE Buffer: 200 mM Tris–HCl, pH 8.0, 200 mM NaCl, 20 mM EDTA.
2. 20 % SDS solution.
3. Phenol–chloroform–isoamyl alcohol solution (25:24:1), pH 7.9.
4. Ice-cold isopropanol.
5. 3 M NaOAc, pH 5.5.
6. 100 % ethanol.
7. Phosphate buffered saline (PBS).
8. TE buffer: 10 mM Tris–HCl, 1 mM EDTA, pH 8.0.
9. Universal qPCR mix (Kapa Biosystems).
10. Liquid nitrogen.

11. Quant-iT PicoGreen DNA Kit (Invitrogen).
12. Agencourt AMPure XP PCR purification kit (Beckman Coulter).

3 Methods

3.1 Equilibration of the Microflora

3.1.1 Cohousing

The coprophagic behavior of mice makes them an ideal model to study the functional consequences of bacterial transfer to mice housed in the same cage. To allow for full equilibration of the composition of the intestinal microflora, the following protocol has proven effective:

1. After weaning, cohouse inflammasome-deficient mice and wild-type mice in a 1:1 ratio.
2. Maintain cohabitation for at least 4 weeks, to allow for complete transfer of the microbiota (*see* **Note 1**).
3. Perform the experiment of interest while keeping the mice cohoused, as separation may lead to return of the original microbiota composition (*see* **Note 2**).

3.1.2 Cross-Fostering

Shortly after birth, the mucosal surfaces of newborns are colonized by maternally transmitted bacteria [9]. In addition, maternal IgA and antimicrobial peptides are transferred to newborns by breastfeeding, further influencing the establishment of intestinal microbial communities [10]. It is therefore possible to equip wild-type animals with microbial communities from inflammasome-deficient mothers and vice versa, using the following protocol:

1. Set up breeding pairs of wild-type and inflammasome-deficient strains simultaneously, so that litters can be expected on the same day.
2. Within 24 h of birth, swap newborns between both breeding pairs, so that wild-type pups will be cross-fostered by inflammasome-deficient dams and vice versa. Swapping of newborn animals between cages should be carried out with sterile forceps. Avoid touching the newborns.
3. After 3 weeks of cross-fostering, the pups can be weaned and the experiment of interest can be carried out while keeping the genotypes matched.

3.1.3 Transfer of Microbial Communities by Oral Gavage

A third way of imposing a foreign microbial community on recipient mice is to orally gavage a suspension of fecal microbiota.

1. Collect fecal pellets into a tube. Alternatively, when sacrificing the donor animal, collect cecal content.
2. Add 50 μL of PBS to each fecal pellet and homogenize manually with a pestle.

3. Filter the sample through a 40 μm cell strainer.
4. Transfer the sample to recipient mouse by oral gavage (*see* **Note 3**).

The three procedures described so far offer possibilities to equilibrate the microflora between wild-type and inflammasome-deficient mice, and to test whether the intestinal bacterial composition influences their predisposition to a certain phenotype (*see* **Note 4**). Often, however, it is of interest to determine the bacterial taxa which are differentially represented in different microbial communities. In the next section, we describe experimental procedures to harvest and process samples for comprehensive analysis of intestinal bacterial communities. We will then provide an overview of currently available sequencing and analysis methods for the study of the microbiome.

3.2 Analyzing Intestinal Microbial Communities

3.2.1 Sampling and Processing of Fecal Material

1. Add 250 μL of 0.1 mm beads to each 2 mL screw-cap tube. Record the weight.
2. Collect fresh feces from mice into collection tubes and freeze immediately in liquid nitrogen.
3. Transfer the sample from the collection tube to a bead-containing tube. This can be done by placing them end-to-end and flipping the sample into the new tube. Record the weight of the full tube. Calculate net weight of sample.
4. Add 500 μL of TNE buffer.
5. Add 210 μL of 20 % SDS.
6. Add 500 μL of phenol–chloroform–isoamyl alcohol solution. Keep samples on ice.
7. Homogenize the sample for 2 min using a mini-bead beater (*see* **Note 5**).
8. Centrifuge the tubes at 5,000 × *g* for 3 min at 4 °C.
9. In the meantime, spin down PLG columns at 14,000 × *g* for 30 s at room temperature.
10. Transfer the aqueous phase (around 600 μL) to the pre-spun PLG tube.
11. Add 600 μL of phenol–chloroform–isoamyl alcohol solution to the sample and mix by inversion (do not vortex).
12. Centrifuge the tubes at 14,000 × *g* for 5 min at room temperature.
13. Transfer the aqueous phase to a new 1.5 mL tube.
14. Add 60 μL (i.e., 10 % of final vol) of 3 M NaOAc and mix thoroughly by inversion.
15. Add 600 μL of ice-cold isopropanol.

16. Store at –20 °C overnight (*see* **Note 6**).
17. Centrifuge tubes at 14,000×*g* for 20 min at 4 °C. Decant supernatant.
18. Wash the pellet (which may range from dark brown to off-white, depending on the diet the animal was on) with 500 μL of 100 % ethanol.
19. Centrifuge for 3 min at 4 °C. Decant the supernatant.
20. Gently remove excess ethanol.
21. Suspend pellet in TE buffer using 5 μL per mg fecal pellet material used in isolation.
22. Incubate for 30 min at 50 °C. Vortex every 10 min to dislodge pellet from tube wall.
23. Store at –20 °C until further use.

3.2.2 Analysis of Selected Members of the Microbiota

The composition of the intestinal microbiome is most commonly assessed by DNA-based approaches, although metabolome, transcriptome, and proteome studies can often be similarly informative [11]. Usually, a marker gene is amplified and the amplicon is then sequenced. Amplicons are commonly between 150 and 500 base pairs in length. The most widely used marker to identify the presence of certain microbial elements in the community of interest is the DNA encoding for the 16S ribosomal RNA subunit (16S rDNA). The following protocol allows for quantifying the amount of specific 16S rDNA in total DNA extracted from fecal material.

1. Choose the PCR primers of interest. These include primer pairs covering total bacteria (such as the widely used pair F8-R338, *see* **Note 7**) as well as those specific for the bacterial taxa of interest. 16S rDNA reference sequences are available in several databases, such as SILVA [12] and the Ribosomal Database Project [13].
2. Mix 5 μL of the sample DNA solution with 5 pmol of each primer.
3. Add 0.2 μL of bacteria-specific probes and 5 μL of universal qPCR mix.
4. Run real-time PCR at 95 °C for 120 s, followed by 40 cycles of 95 °C for 3 s and 64 °C for 30 s.
5. Quantify abundance of bacterial taxa by normalizing to signal obtained for total bacterial 16S sequences (*see* **Note 8**).

3.2.3 Comprehensive Sequencing Analysis of Microbial Communities

The realization that the microbiome is controlled by components of the innate immune system and that the composition of intestinal microbial communities substantially affects many aspects of the host's physiology has precipitated the development of technologies for the fast collection of microbiome profiles at relatively low cost.

Experimental tools for such profiling studies have been reviewed in depth recently [14], and we will focus here on the general framework and basic steps necessary for the determination of the bacterial microbiome of inflammasome-deficient mice by 16S rDNA sequencing.

1. Carefully choose the PCR primers for amplification (*see* **Note 9**).
2. Perform three replicate PCRs for each fecal DNA.
3. Pool the three reactions in an equimolar ratio after DNA quantification with the PicoGreen DNA Kit.
4. Purify the DNA with the Ampure magnetic purification beads.
5. Obtain sequences from isolated bacterial DNA (*see* **Note 10**).
6. After acquiring DNA sequences, the errors that have been produced during the sequencing process need to be removed by clustering the obtained sequences. Useful bioinformatic tools for this step are Denoiser and AmpliconNoise [15].
7. Next, process the reads using bioinformatic tools which allow for comparative analysis of microbial taxa based on targeted amplicon sequences, such as QIIME [16] or mothur [17]. These programs evaluate the amplicons based on sequence similarity and cluster them into operational taxonomic units (OTUs), usually binning sequences sharing at least 97 % nucleotide sequence identity into OTUs.
8. To extract phylogenetic information, reference databases such as SILVA [12] or greengenes [18] can be used and the obtained phylogenies can be interpreted using analysis tools such as UniFrac [19] (*see* **Note 11**).

4 Notes

1. Cohousing should ideally start immediately after weaning, i.e., at about 3 weeks of age, and should be maintained until the experiment is performed. A minimum of 4 weeks of cohousing needs to be strictly kept to avoid partial transmission of the microbiota [20]. Changes of cage bedding and washing of cages can be routinely performed during the cohousing period. Due to their less aggressive behavior, female C57BL/6 mice are generally more suitable for cohousing experiments. However, when cohoused immediately after weaning, male mice can also be used.
2. Separation of previously cohoused mice leads to the successive loss of the "foreign" microflora composition [5], supporting the concept that the host genotype defines the availability of niches for intestinal microbial colonization.

3. In order to increase efficiency of bacterial transfer, mice may be starved for 6 h before gavage. When performing the gavage, hyperextend the neck of the mouse and slowly insert the gavage needle to avoid injury when delivering material into the stomach. Mice should not be anesthetized for this procedure.
4. In our experience, 4 weeks of cohousing is the most reliable way to ensure full equilibration of the microflora between two genotypes of interest. Cross-fostering may be devised to analyze for the effect of maternal transmission to newborns. Oral gavage of fecal homogenates is most efficient when the recipient mouse does not harbor a full complex microbiota, such as germ-free mice or antibiotic-pretreated mice. Otherwise, the indigenous flora provides colonization resistance against incoming bacteria [21]. The latter method is most useful to introduce defined members of the microbial community, while cohousing and cross-fostering will change the entirety of the intestinal microbiota composition over a longer period of time.

   The use of heterozygous breeding is an efficient way of ensuring an equilibrated microbiota composition between inflammasome knockout mice and their wild-type or heterozygous littermates. In this setting, to test for a role of genotype-dependent dysbiosis, inflammasome-deficient mice and their littermates should be separated upon weaning and housed in separate cages for several weeks, allowing for divergent development of microflora compositions based on the genetic makeup of the respective hosts. In the experimental setup of interest, these separately housed animals should be compared to non-separated knockouts and their littermates, to assess whether the spatial separation lead to the establishment of different intestinal microbiota compositions.
5. If bead beating technology is unavailable, extensive vortexing and manual homogenization with a pestle under sterile conditions might provide a suitable alternative.
6. The overnight precipitation may be used as a break point in the protocol. However, in our experience, 1 h at –20 °C is already sufficient for full precipitation efficiency.
7. Primer sequences: F8 5′-AGAGTTTGATCCTGGCTCAG-3′; R338 5′-TGCTGCCTCCCGTAGGAGT-3′.
8. Dysbiosis in inflammasome-deficient mice has been associated with an outgrowth of *Prevotellaceae* and *TM7*, and an underrepresentation of *Lactobacillaceae* [5]. These taxa might therefore serve as a useful starting point when targeting aberrantly represented members of the bacterial community in inflammasome-deficient mice by qPCR.
9. The available primer pairs vary greatly with respect to compatibility with various sequencing instruments, taxonomic coverage, and the depth of phylogenetic specificity. The above mentioned primer pair F27-R338, for instance, albeit useful

for mapping the entire bacterial community, biases against certain groups [14].

10. To date, several sequencing platforms are available which devise various technological principles, including pyrosequencing (such as the Roche 454 Sequencer, FLX and FLX Titanium), clonal arrays (e.g., HiSeq 2000, Illumina), and capillary sequencing (such as ABI 3730). The individual platforms have different performance features with respect to speed, error rate, cost, and read length.
11. Once assigned to OTUs and analyzed by UniFrac, 16S rDNA sequencing data is still fairly extensive and hard to interpret. To facilitate visual analysis, the data is typically depicted using principal component analysis (PCoA), which identifies common relationships among microbial communities and visualizes them in a two- or three-dimensional space. In a PCoA plot, each community is represented by a dot, with mice with a similar microbiota composition being presented close to each other.

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