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Research paper

Induction of Th17 cells by segmented filamentous bacteria in the murine intestine

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ABSTRACT

Segmented filamentous bacteria (SFB) are Gram-positive, anaerobic, spore-forming commensals that reside in the gut of many animal species. Described more than forty years ago, SFB have recently gained interest due to their unique ability to modulate the host immune system through induction of IgA and Th17 cells. Here, we describe a collection of methods to detect and quantify SFB and SFB adhesion in intestinal mucosa, as well as SFB-specific CD4 T cells in the lamina propria. In addition, we describe methods for purification of SFB from fecal material of SFB-monoassociated gnotobiotic mice. Using these methods we examine the kinetics of SFB colonization and Th17 cell induction. We also show that SFB colonize unevenly the intestinal mucosa and that SFB adherence occurs predominantly in the terminal ileum and correlates with an increased proportion of SFB-specific Th17 cells.

1. Introduction

Segmented filamentous bacteria (SFB, *Candidatus Arthromitus* or *Candidatus Savagella*), are members of the resident murine flora, most closely related to Clostridia (Prakash et al., 2011; Sczesnak et al., 2011). SFB have been described in many vertebrate and invertebrate animal species, as well as in humans (Klaasen et al., 1993; Yin et al., 2013). In contrast to most other commensals, SFB interact directly with intestinal epithelial cells (IECs) in the terminal ileum (Blumershine and Savage, 1978; Klaasen et al., 1992). In contrast to invasive pathogens, SFB do not invade epithelial cells, penetrate the epithelial barrier, or induce intestinal inflammation (Talham et al., 1999). SFB are currently propagated exclusively in SFB-monoassociated gnotobiotic animals (SFB-mono mice) (Klaasen et al., 1991; Umesaki et al., 1995). However, a recent exciting study has described a method for *in vitro* propagation of the bacteria that promises to allow genetic manipulation of this microorganism in the near future (Schnupf et al., 2015). The original studies in SFB-mono mice showed that SFB have immunomodulatory effects, such as induction of IgA production, recruitment of IELs, and induction of MHCII expression and glycosylation in IECs (Talham et al., 1999; Umesaki et al., 1995), although it remains unclear how specific these functions are to SFB. More recently, SFB were shown to be a specific inducer of Th17 cell differentiation (Ivanov et al., 2009). Even though SFB colonization leads to a general recruitment of CD4 T cells, Th17 cells are the only CD4 T cell subset that is proportionately increased with SFB colonization (Gaboriau-Routhiau et al., 2009; Ivanov et al., 2009). Characterization of the specificity of the Th17 cell response in SFB-colonized mice, revealed that most intestinal Th17 cells are SFB-specific and that virtually all SFB-specific T

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cells are Th17 cells (Goto et al., 2014; Yang et al., 2014). Thus, unlike other commensals, SFB are capable of inducing a localized antigen-specific Th17 response. Because of the unique nature of this response, as well as the unique nature of the interaction between SFB and the host, SFB are an excellent model for elucidating fundamental cellular and molecular mechanisms of Th17 cell induction and the effects of mucosa-associated bacteria on host immunity.

2. Materials and methods

2.1. Mice

The mice used in this study are C57BL/6 mice obtained from location MP14 at the Jackson laboratory (JAX) and location IBU7 at Taconic Farms (TAC). We have previously reported that JAX mice lack SFB and TAC mice contain SFB as part of their resident microbiota (Ivanov et al., 2009). However, the presence of SFB depends on the particular barrier location at each vendor. Indeed, mice from certain locations at JAX contain SFB and mice from certain locations at TAC do not. Therefore, the presence or absence of SFB cannot be assumed based solely on vendor source, and we test every animal for SFB upon arrival using the methods described below. Of note, Taconic Farms has recently implemented quarterly screens for SFB as part of their health monitoring program and the results can be found on the corresponding health reports on the Taconic website.

2.2. Colonization of mice with SFB by oral gavage

Fecal pellets for oral gavage are collected from live SFB-positive mice and frozen at −80 °C until use. Alternatively, pellets may be collected and homogenized immediately for gavage as described below. Frozen feces are transported to the vivarium on dry ice and homogenized in water using a 20 G needle. Solids are separated by gravity before the cleared supernatant is transferred to a 50 ml conical tube. Cleared supernatants are introduced into mice (200–500 μl/animal) by oral gavage. We generally use the equivalent of the contents of 1–2 fecal pellets per recipient mouse. In our hands this procedure results in colonization of most animals when using feces from SFB-positive specific pathogen free (SPF) mice (90–100%). For better results, especially when using feces from SFB-mono mice, the gavage can be repeated after 4–8 h (colonization with SFB-mono feces after a single gavage can be variable and may depend on host genetics and endogenous microbiota). In all cases, SFB colonization must be confirmed by qPCR. In successfully colonized animals, SFB can be detected in feces on day 4 post-colonization. We usually confirm SFB levels by qPCR on day 4 and day 8 after the last gavage.

2.3. Genomic DNA extraction from feces and quantitative real-time PCR (qPCR)

Fresh or frozen fecal pellets are weighed prior to DNA extraction. Individual fecal pellets are weighed and placed in 2 ml polypropylene tubes (Sarstedt) together with 0.5 ml of 0.1 mm zirconia beads (BioSpec), 500 μl of DNA extraction buffer (200 mM Tris, 200 mM NaCl and 20 mM EDTA), 210 μl of 20% SDS and 500 μl of Phenol–Chloroform–Isoamyl alcohol (P:C:I) (25:24:1). Bacterial cells in the sample are lysed using a FastPrep 24 bead-beater (MP Biomedical) for 1 min at maximum speed. Note that the addition of more than 100 mg of feces results in incomplete lysis and extraction of bacterial DNA and therefore we do not recommend using more than a single fecal pellet per tube. After lysis, the samples are centrifuged at 16,000 *g* for 5 min at 4 °C and the DNA-containing aqueous phase is collected and subjected to two additional P:C:I extractions to further remove organic contaminants. DNA is precipitated with 0.1 volume 3 M sodium acetate, pH 5.2 and two volumes of 100% ethanol at −80 °C for 10–20 min followed by centrifugation for 20 min at 16,000 *g* and 4 °C. After washing in 70% ethanol, the DNA pellet is resuspended in low strength Tris–EDTA buffer. Contaminating RNA is removed by treatment with 0.1 mg/ml of RNAse A (Thermo Fisher) for 5 min at room temperature (RT).

qPCR is conducted on the fecal DNA to determine the amount of SFB and total Eubacteria present in each sample by amplifying 16S rRNA genes using the following bacteria-specific primers (Barman et al., 2008): 5′-GACGCTGAGGCATGAGAGCAT-3′ and 5′-GACGGCACGGATTGTTATTCA-3′ for SFB, and 5′-ACTC CTACGGGAGGCAGCAGT-3′ and 5′-ATTACCGCGGCTGCTGCG-3′ for Eubacteria (UNI). qPCR is performed on a Roche LightCycler 480 with SYBR Green reagent. The PCR conditions include a pre-incubation step at 95° for 5 min, 40 amplification cycles of 95 °C for 10 s and annealing at 62 °C for 45 s, and a melting curve.

2.4. Isolation of mucosa-associated bacteria

A 0.5 cm piece of the intestine is removed after sacrifice, opened longitudinally and flushed vigorously with sterile PBS to remove mucus, intestinal contents and any loosely associated bacteria before being placed in cold, sterile PBS. The epithelial layer is loosened by incubation in cold 30 mM EDTA in PBS or HBSS for 15 min. The tissue is then placed in cold sterile PBS and the epithelial layer is separated from the underlying basal membrane and muscularis mucosae by gently scraping with a bent sterile 28 G needle under a dissecting microscope. The epithelial sheets containing mucosa-associated bacteria are collected and subjected to genomic DNA extraction as described in Section 2.3

2.5. Assessing SFB-specific T cell responses using an ex vivo proliferation assay

Lamina propria lymphocytes (LPL) are isolated as previously described (Goto et al., 2014). CD4 T cells are purified from this preparation by fluorescence-activated cell sorting (FACS) or magnetic beads (MACS) and labeled with CFSE or CellTrace Violet proliferation dye (Life Technologies). For assessment of SFB-specific responses, 5×10^4 purified CD4 T cells are co-cultured in T cell medium (RPMI containing 10% FCS, 1% non-essential amino acids, 1% sodium pyruvate, 1% L-glutamine, 1% Pen/Strep, 20 mM HEPES and 0.001% β-mercaptoethanol) in 96 well U-bottom plates with either 5×10^4 MACS purified splenic $CD11c^+$ dendritic cells or 2×10^5 total TCRα-KO splenocytes as antigen presenting cells in the presence or absence of autoclaved bacterial lysates (prepared as described in Section 2.6). Specific proliferation in response to presented antigens is determined by flow cytometry at 72 h by calculating the number of live CD4 T cells with dye dilution or of live blasting (FSC^{high}) CD4 T cells.

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