Comparative Immunology, Microbiology and Infectious Diseases 36 (2013) 149–160

Contents lists available at SciVerse ScienceDirect

Comparative Immunology, Microbiology and Infectious Diseases

journal homepage: www.elsevier.com/locate/cimid

Exploring the immune response of porcine mesenteric lymph nodes to *Salmonella enterica* serovar Typhimurium: an analysis of transcriptional changes, morphological alterations and pathogen burden

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ARTICLE INFO

Article history:
Received 22 May 2012
Received in revised form
14 November 2012
Accepted 19 November 2012

Keywords:
Pig
Mesenteric lymph-node
Immune response
Salmonella enterica serovar Typhimurium

ABSTRACT

Infections caused by *Salmonella* enterica serovar Typhimurium (*S. typhimurium*) cause important economic problems in the swine industry and threaten the integrity of a safe and healthy food supply. Controlling the prevalence of *Salmonella* in pig production requires a thorough knowledge of the response processes that occurs in the gut associated immune tissues. To explore the *in vivo* porcine response to *S. typhimurium*, MLN samples from four control pigs and twelve infected animals at 1, 2 and 6 days post infection (dpi) were collected to quantify the mRNA expression of gene coding for 42 innate immune-related molecules. In addition, the presence of *S. typhimurium* in MLN was examined and its effect on tissue micro-anatomy. Higher *S. typhimurium* loads were observed at 2 dpi, triggering an innate immune response, marked by a substantial infiltration of phagocytes and up-regulation of pro-inflammatory genes. Such response resulted in a significant decrease in pathogen burden in MLN at 6 dpi, although *Salmonella* could not be completely eliminated from tissue. Furthermore, our results suggest that in porcine infections, *S. typhimurium* might interferes with dendritic cell–T cell interactions and this strategy could be involved in the conversion of *Salmonella* infected pigs to a carrier state.

1. Introduction

Salmonella is one of the most frequent causes of food-borne outbreaks in Europe, with 108,614 confirmed cases of human salmonellosis in 2009 [1]. Worldwide, *Salmonella* causes 94 million cases of acute gastroenteritis, including 155,000 that are fatal, with children in particular falling victim to the disease [2]. Among more than 2500 serovariants, the host-generalist *Salmonella enterica* serovar Typhimurium (herein *S. typhimurium*) is reported as the serovar most frequently associated with human illness, with *S. typhimurium* cases mostly associated with the consumption of contaminated pig and poultry meat [3].

Infections by *S. typhimurium* in pigs lead to a localized enterocolitis and, in general, infected swine evolve into healthy carriers in which bacteria are able to persist without triggering clinical signs [3]. The existence of asymptomatic carriers is a major threat to public health

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http://dx.doi.org/10.1016/j.cimid.2012.11.003

given that such animals cannot be detected easily and thus serves as source of contamination in food industry [4]. In addition to its importance as zoonotic disease, salmonellosis has an important impact in porcine health and negative implications in the efficiency and economy of the porcine production systems [5].

As a result of food poisoning, *Salmonella* enters the body through the gastrointestinal tract. After location in intestinal lumen and attachment in epithelial cells, *Salmonella* actively invade intestinal cells, colonize the lamina propria and Peyer's patches and rapidly invade host cells, especially macrophages, but also dendritic cells and neutrophils [6]. Consequently, these innate immune cells produce and release chemoattractant cytokines to recruit additional inflammatory cells into the site of invasion and initiate a T helper 1 (Th1) response [7]. From intestine, *Salmonella* reaches mesenteric lymph nodes (MLN), can enter the bloodstream and spread to internal organs [8]. To prevent systemic infection, MLN form a life-saving firewall that protects the host from rapid pathogen dissemination beyond the intestine to others organs, such as liver and spleen [9].

One way to learn about the molecular interactions during *Salmonella* infection is to analyze the host response. To this end, infection experiments using isolated primary cells or cell lines have been carried out to generate most of the knowledge currently available on the molecular events during *Salmonella*–host interaction [10–12]. However, although these *in vitro* models can provide valuable information, it is clear that this approach does not enable the interaction of pathogens with a multitude of different interacting cells involved in the invasion process *in vivo* [13]. Moreover, in mammals, studies on the host mechanisms against *Salmonella* have been largely focused on mice infection models [14,15], although accumulating evidences suggest differences in virulence mechanisms, pathogen colonization and disease susceptibility in food-producing animals infections compared to the murine model of systemic disease [16]. Thus, while in mice *S. typhimurium* moves into the mesenteric lymph nodes, and from there bacteria spread *via* the efferent lymph to the circulatory system, causing a systemic disease [17], in pigs usually causes a self-limiting intestinal disease enterocolitis which is similar to gastroenteritis in humans [6]. Factors that could cause these differences have not been sufficiently clarified, but make the pig an ideal model for investigating enteric salmonellosis in humans.

In swine, *in vitro* and *in vivo* studies have generated valuable insight in the crosstalk between *Salmonella* and porcine tissues and cells [18–20]. Nevertheless, few studies have addressed the requirements for the gut-associated lymph nodes in the development of immune responses, their effect on the protective immunity against *Salmonella* infection and the relationship between changes in MLN transcriptome, tissue cellularity and level of pathogen invasion. In order to contribute to improved knowledge on the role of porcine MLN on *S. typhimurium* infection, in the current study we use a model of *in vivo* experimental challenge to evaluate changes in gene expression and tissue morphological alterations that occur in this organ following infection.

2. Materials and methods

2.1. Experimental infection

The experimental infection design was described elsewhere [19]. Briefly, sixteen weaned piglets of approximately 4 weeks of age and fecal-negative for *Salmonella* were penned in an environmentally controlled isolation facility at 25 °C with *ad libitum* access to feed and water. All the animals were randomly allocated to control group (4 piglets) or infected group (12 piglets). The four non-infected pigs were necropsied 2 h before the experimental infection. The animals belonging to the infected group were orally challenged with 10^8 CFU of *S. typhimurium* phagetype DT104. Afterwards, four infected pigs were randomly sampled and necropsied at 1, 2 and 6 days post infection (dpi). Fecal samples from the infected group were cultured for *Salmonella* to ensure the effectiveness of the experimental challenge. Furthermore, rectal temperature and clinical signs were daily recorded for each animal to observe the evolution of the infection. All procedures involving animals were performed in accordance with the European regulations regarding the protection of animals used for experimental and other scientific purposes.

2.2. Histopathology and immunohistochemistry

Samples of MLN from all experimental animals were fixed in 10% neutral buffered formalin for 24 h and embedded in paraffin-wax for histological processing. Afterwards, 5 μm tissue sections were routinely processed as previously described [21], and stained with hematoxylin and eosin (H&E). Presence of bacteria in the tissue samples was demonstrated by using an anti-*Salmonella* polyclonal antibody raised by immunization of a New Zealand rabbit with a formalin fixed bacterial suspension. Quantification of tissue infiltration of macrophages was carried out by using a monoclonal antibody specific for porcine macrophages (clone 4E9/11) [22]. Immunohistochemical staining of formalin-fixed, paraffin-embedded sections of porcine MLN samples, subjected to heat-mediated antigen retrieval in 0.01 M citric acid, with *Salmonella* antiserum and 4E9 monoclonal antibody was performed by using the immunoperoxidase method as has been described elsewhere [21]. Neutrophils identify by morphology and immunolabeled macrophages were counted in 50 randomly selected high magnification fields (400×) in sections of two different MLN samples from each infected and control animal. Results were expressed as the mean number of cells per field.

2.3. Nucleic acids purification

Samples of MLN from control and infected animals were aseptically collected after necropsies and immediately frozen in liquid nitrogen for DNA and RNA isolation. Then, after treatment with RNA*later*-ICE (Ambion), a volume of 0.6 ml of RLT buffer (Qiagen) was added per 30 mg of tissue followed by disruption in a rotor–stator homogenizer. DNA and RNA were isolated by using the AllPrep DNA/RNA/Protein Mini Kit (Qiagen) and eluted RNA was