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Short communication

Innate and adaptive immune mechanisms are effectively induced in ileal Peyer's patches of *Salmonella typhimurium* infected pigs

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ABSTRACT

In this report we employed laser-capture microdissection (LCM) coupled to qPCR technology and bioinformatic analysis to characterize, for the first time, the response of Peyer's patches (PP) from orally infected animals to *Salmonella typhimurium*, in a model of non-typhoidal salmonellosis. Pathogen was highly found in the cytoplasm of phagocytes in PP and differential gene expression analysis indicated an up-regulation of proinflammatory molecules, establishment of a Th1 driven response and triggering of DC and T-cell activity. Furthermore, predictions by bioinformatic analysis pointed to an activation of processes regarding stimulation and maturation of DC, influx of leukocytes in tissue and T lymphocytes priming and differentiation. In short, the approach used in this study proved to be a promising strategy to explore infectious processes. Indeed, it revealed an effective induction of innate and adaptive immune mechanisms in swine PP which appear to be distinct from those observed in mesenteric lymph nodes and closely related to response of gut mucosa.

1. Introduction

Despite the current improvement in sanitation and hygiene, *Salmonella* persists as a significant cause of disease worldwide. Data from the Centers for Disease Control (CDC) assert that *Salmonella* alone causes approximately 1 million foodborne infections annually in the United States (CDC, 2011). Similarly, *Salmonella* is reported as the main cause of food-borne outbreaks in the European Union, being *Salmonella enterica* serovar *Typhimurium* (herein *Salmonella typhimurium*) the second most frequently isolated serovar from human infections (EFSA, 2012).

Advances in mammalian models of *Salmonella* infection are expected to result in new understanding of salmonellosis pathogenesis, contributing to the control and cure of human cases (Gopinath et al., 2012). In this context, pigs can be stressed as an ideal model for investigating human non-typhoidal salmonellosis, since upon infection with *S. typhimurium* swine undergo a self-limiting enterocolitis similar to the clinical manifestation observed in man.

Studies on the murine model of typhoid fever state that to cause infection, ingested *S. typhimurium* primarily invade M cells in the small intestine and then accesses Peyer's patches (PP), resulting in a massive inflammatory response in these organs (Broz et al., 2012). Therefore, besides their role in the immune surveillance of the intestinal lumen, PP are relevant mediators of infections by *S. typhimurium*. Although previous studies have employed gut loops models to extrapolate the response of PP to *S. typhimurium* (Meurens et al., 2009; Nunes et al., 2010), the function of these organs in the context of oral non-typhoidal infections has never been explored to date. To address this issue, we employed for the first time laser-capture microdissection (LCM) coupled to qPCR technology and bioinformatic analysis to provide an accurate view of the immune mechanisms modulated in PP of pigs orally infected with *S. typhimurium*.

2. Material and methods

2.1. Experimental infection

Eight crossbred piglets of approximately four weeks of age, confirmed to be fecal-negative for *Salmonella*, were randomly allocated to control or infected groups (four animals each), being control (0 day post-infection — dpi) pigs necropsied 2 h before the experimental infection. Pigs belonging to the infected group were orally challenged with 10^8 cfu of *S. typhimurium* phagetype DT104 and euthanized at 2 dpi. Ileum samples were collected from all piglets and immediately frozen in liquid nitrogen for RNA isolation or fixed in 10% neutral buffered formalin for immunohistochemistry

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assays. All procedures involving animals were performed under the supervision of the Ethical and Animal Welfare Committee of the University of León (Spain), in accordance with the European regulations regarding the protection of animals used for experimental and other scientific purposes.

2.2. Laser-capture microdissection and RNA preparations

Frozen gut samples from all experimental animals were embedded in optimal cutting temperature compound (Sakura Finetek USA, Torrance, CA, USA) and cut into serial 20 μm sections. Before microdissection, eight cryostat sections from each pig were mounted on glass slides and treated with RNAlater-ICE (Ambion) according to manufacturer instructions. Subsequently, PP follicles were laser-microdissected and captured from terminal ileum sections avoiding contamination by adjacent cells with a PALM MicroBeam device (Carl Zeiss MicroImaging GmbH, Jena, Germany), by Auto-Laser Pressure Catapulting (LPC) mode (Fig. 1A–C). Catapulted tissue was soaked in 15 μl of RLT buffer (Qiagen, Valencia, CA, USA) poured in 200 μl microtubes caps and RNA purifications were carried out employing the RNeasy Mini Kit (Qiagen). Eluted RNA was digested with RNase-Free DNase Set (Qiagen) and RNA quality was checked by Experion RNA analysis (Bio-Rad, Hercules, CA, USA). Finally, RNA was amplified using the SuperScript™ RNA Amplification System (Invitrogen, Carlsbad, CA, USA), as indicated by manufacturer.

2.3. Real-time quantitative PCR (qPCR)

Amplified RNA from infected and control animals was reverse transcribed to cDNA using the qScript cDNA Synthesis kit (Quanta BioSciences, Gaithersburg, MD, USA) and qPCR assays were performed according to Martins et al. (2013) to determine the relative expression of 30 genes coding for molecules taking part in distinct immune response processes such as inflammation, DC-T cell interaction and T helper cell response. Primers used for amplifications can be found as supporting information (see Supplementary File 1). Afterwards, relative gene expression was assessed by the $2^{-\Delta\Delta Cq}$ method (Livak and Schmittgen, 2001). In this analysis, qPCR data were presented as the fold change in gene expression normalized to an endogenous reference gene and relative to the uninfected controls. Fold change values higher than 1 meant up-regulation. Values inferior to 1 were calculated as −1/fold change and denoted down-regulation. Data were analyzed by Student's *t*-test using the software SPSS 15.0 for Windows® (SPSS, Inc). A *p*-value bellow 0.05 was considered statistically significant.

2.4. Bioinformatic data analysis

Ingenuity Pathway Analysis (IPA, Ingenuity Systems, www.ingenuity.com) Downstream Effects tool was used to identify functions that are expected to be activated in tissue, given the observed gene expression patterns. Predictions were made by *z*-score algorithm and values higher and lower than 2 meant that activation state was statistically increased and decreased respectively. IPA Path Designer was also employed to illustrate some mechanisms modulated by genes evaluated in this study.

2.5. Immunohistochemistry

To verify the presence of *S. typhimurium* in PP, paraffin sections (5 μm) of formalin fixed samples were routinely processed and immunostained as described elsewhere (Martins et al., 2013), employing a specific anti-*Salmonella* rabbit antiserum.

3. Results and discussion

Ileal loops models have provided valuable information regarding the response of gut mucosa to *Salmonella*. Nevertheless, accumulating evidences indicate that ingested *Salmonella* undergo phenotypical changes during its passage along host gastrointestinal tract that influence the infectious process (Alvarez-Ordóñez et al., 2011). Thus, it could be speculated that infection conditions employed by this approach is substantially different from those found in natural infections. Basing on this, in this report we describe for the first time the regulation of immune response mechanisms in the PP of orally infected animals, in the context of non-typhoidal salmonellosis. Besides, LCM was used to isolate and analyze cell exclusively from PP follicles. Microdissection has been successfully employed in cancer research to provide precise knowledge on tumor biology (Cheng et al., 2013). Here, LCM coupled to qPCR technology enabled us to characterize changes in infected PP with reduced interference from non-target cells, generating more accurate data than previously reported.

Immunohistochemistry assays demonstrated that *S. typhimurium* was notably found in PP (Fig. 1D and E), agreeing with preceding reports that highlight these organs as the main portal of pathogen entry to host mucosa (Broz et al., 2012; Schauser et al., 2004). Moreover, differential expression analysis revealed significant regulation of 21 out 30 genes encoding pattern-recognition receptors (PRR), chemokines, DC and T-cell activation markers, Th response mediators and other immune-related molecules (Table 1). Proinflammatory genes such as IL1β, CXCL2 and TNFα were found to be up-regulated as a consequence of infection. This response could be associated to recognition of invading *Salmonella* via PRR by macrophages and dendritic cells in PP, resulting in pathogen phagocytosis, secretion of chemokines and recruitment of additional inflammatory cells into the site of invasion (Broz et al., 2012). In line with this, a strong up-regulation was uncovered for all screened PRR (TLR2, 4, 5, 8 and NOD2) and pathogen labeling was mainly observed in the cytoplasm of large irregular-shaped mononuclear cells and some polymorphonuclear cells (Fig. 1F). Inflammation induced by IL1 has been considered a key mechanism in *Salmonella* pathogenesis at mucosa level (Raupach et al., 2006). However, a previous report employing porcine gut loops did not detect up-regulation of IL1β and chemokines encoding genes in PP upon *S. typhimurium* infection (Meurens et al., 2009). Although we believe that this discrepancy could be attributed to differences between approaches, PP areas not analyzed by us, such as dome and interfollicular zones, might affect the response of these organs to infection. Thus, this fact could also justify the observed differences between current study and others on the whole PP.

In general, results indicated that similarly to the murine typhoid model (Tam et al., 2008), mechanisms of innate immune response are effectively induced in PP during swine oral infections by *S. typhimurium*. Apart from contributing to bacterial clearance in tissue, as suggested by our previous observations (Martins et al., 2013), this response could be related to triggering of host second line of defense. Genes coding for molecules involved in DC activation (CD80, CD83, CD40, IL12p40, IL23p19 and CCR7) were found to be up-regulated in infected PP (Supplementary File 2). The same was observed for molecules required for T-cell mediated processes (IFNγ, CD40L, CD28, CCL19 and IL21). Of note, high mRNA levels were observed for IFNγ and IL12p40, despite the absence of IL18 up-regulation. Although this interleukin act synergistically with IL12 in the induction of IFNγ production and cell-mediated immunity, a previous study demonstrated that IL-18 is relevant for resistance to the systemic infection but not during the intestinal phase of salmonellosis (Raupach et al., 2006). Concurrently, down-regulation or absence of expression was uncovered for Th2 (IL4 and IL13)