

## Increased number of intestinal villous M cells in levamisole - pretreated weaned pigs experimentally infected with F4ac<sup>+</sup> enterotoxigenic *Escherichia coli* strain

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### Abstract

Immunoprophylaxis of porcine postweaning colibacillosis (PWC) caused by enterotoxigenic *Escherichia coli* (ETEC) expressing F4 fimbriae is an unsolved problem. Just as ETEC strains can exploit intestinal microfold (M) cells as the entry portal for infection, their high transcytotic ability make them an attractive target for mucosally delivered vaccines, adjuvants and therapeutics. We have developed a model of parenteral/oral immunization of 4-weeks-old pigs with either levamisole or vaccine candidate F4ac<sup>+</sup> non-ETEC strain to study their effects on *de novo* differentiation of antigen-sampling M cells. Identification, localization and morphometric quantification of cytokeratin 18 positive M cells in the ileal mucosa of 6-weeks-old pigs revealed that they were: 1) exclusively located within villous epithelial layer, 2) significantly numerous ( $P < 0.01$ ) in levamisole pretreated/challenged pigs, and 3) only slightly, but not significantly numerous in vaccinated/challenged pigs compared with non-pretreated/challenged control pigs. The fact that levamisole may affect the M cells frequency by increasing their numbers, makes it an interesting adjuvant to study development of an effective M cell-targeted vaccine against porcine PWC.

### Introduction

Immunoprophylaxis of postweaning colibacillosis (PWC) is an unsolved problem. Up today, the most promising vaccination strategies include oral immunization of pigs before weaning with live avirulent non-enterotoxigenic *Escherichia coli* (non-ETEC) strains carrying fimbrial adhesins homologous to those of ETEC strains present in the given swine population.<sup>1</sup> However, the immunization against PWC with live vaccines is still difficult, for the following reasons: i) ETEC strains are only one of the essential factors of the etiology of PWC<sup>2</sup> ii) the success of immunization depend on the presence of intestinal receptors specific for ETEC adhesins<sup>3</sup> and on the uptake of bacterial antigens by the microfold (M) cells, specialized extensions of germinal centers of Peyer's patches<sup>4</sup> iii) the small intestinal mucosal immune system may develop different immune responses to the same antigens depending on the dose and form of immunogen, resulting in production of protective IgA, oral tolerance or hypersensitivity.<sup>5,6</sup> To avoid induction of oral tolerance in developing pigs,<sup>7</sup> the immunization strategies to control this disease include the use of adjuvants, such as levamisole.<sup>8,9</sup> The attempts to validate the influence of levamisole on the intestinal cellular immunity against PWC, when applied alone or in the combination with live oral F4ac<sup>+</sup> or F18ac<sup>+</sup> vaccines, confirmed its potentials as an immunomodulator and mucosal adjuvant.<sup>10,11</sup> However, our knowledge about mechanisms operating at the mucosal surfaces of the small intestine following oral immunization of pigs with a live vaccine candidate F4ac<sup>+</sup> non-ETEC strain is still limited. Further research should include antigen sampling microfold (M) cells providing transcytotic antigen delivery through the follicle-associated epithelium (FAE) to the underlying immune cells within the gut associated lymphoid tissues (GALT), such as ileal Peyer's patches.<sup>12,13</sup> Indeed, it has been demonstrated that M cells can transport a diverse array of intraluminal microorganisms across the intestinal epithelial barrier, including *E. coli*,<sup>12</sup> but also a variety of synthetic microparticles/nanoparticles as vehicles for mucosal vaccine delivery,  $\beta$  1 integrins, pathogen recognition receptors and specific carbohydrate residues.<sup>14</sup> Accordingly, some intestinal pathogens exploit M cells as their entry portal to invade host and cause infections.<sup>15</sup> For example, the rabbit diarrheagenic *E. coli* strain is selective for adherence to intestinal M cells.<sup>12</sup> We believe that both porcine ETEC strains and vaccine candidate non-ETEC strains utilize these cells as a target for entry/delivery to induce either an infection or protective immunity, respectively. Porcine

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intestinal M cells may be distinguished from the other cells residing epithelium by the expression of cytokeratin peptide 18.<sup>16</sup>

The current study evaluated nonspecific immunogenic potentials of levamisole in promoting differentiation of porcine ileal M cells phenotype as an attractive target for mucosally delivered vaccines against porcine PWC. Therefore, we have performed identification, localization and morphometric quantification of cytokeratin 18 positive M cells in the ileal mucosa of weaned pigs immunized with either levamisole or vaccine candidate F4ac<sup>+</sup> non-ETEC strain and challenged with homologous ETEC strain.

### Materials and Methods

Fifteen crossbred pigs (Swedish Landrace x Yorkshire) weighed 6.5±1 kg, progeny of three litters, were purchased from a swine farm nearby Zagreb, Croatia. The pigs were weaned at 4 weeks of age, housed in the animal facility at the Veterinary Faculty University of Zagreb and fed with a standard weaner diet. Experimental and animal management procedures were conducted in accordance with the "Directive for the Protection of Vertebrate Animals used for Experimental and other Purposes" (86/609/EEC). The F4ac<sup>+</sup> *E. coli* strains used for either immunization or challenge infection were described in a previous study of our group.<sup>11</sup> Mouse IgG1 (clone C-04) monoclonal antibody (mAb), reactive with

porcine cytokeratin peptide 18<sup>16</sup> was used as primary antibody (Biovendor Laboratory Medicine, Heidelberg, Germany). Polyclonal F(ab')<sub>2</sub> rabbit anti mouse IgG:HRP antibody (AbD Serotec, Oxford, UK) was used as secondary antibody. For uncovering of tissue antigens fixed with formalin, we used a stabilized proteolytic enzyme mixture in an antigen retrieval kit (Abcam, Cambridge, UK), prior the application of primary antibody.

At age of 4 weeks, the pigs were randomly assigned into three groups comprising 5 animals each. After 2 days of accommodation, pigs were treated as follows: i) control pigs received 5 mL of saline intramuscularly (*i.m.*), at day 0; ii) pigs from the first group of principals were *i.m.* primed with levamisole (Nilverm®; Pliva, Zagreb, Croatia) at the immunostimulatory dose of 2.5 mg/kg over three consecutive days (-2, -1, 0); and iii) the second group of principals were intragastrically (*i.g.*) immunized with 10<sup>10</sup> CFU/mL of F4ac<sup>+</sup> non-EPEC vaccine candidate strain 2407 in 60 mL of Trypticase soya bujon (TSB), at day 0. Seven days later all pigs were *i.g.* challenged with 10<sup>10</sup> CFU/mL of F4ac<sup>+</sup> EPEC strain 11-800/1/94 and two out of each group were euthanized at day 13 and sampled for immunohistology.

Immediately following euthanasia, the samples of ileum were fixed in 10% neutral-buffered formalin (pH 7.0-7.6) containing 4% formaldehyde for 24 hours. Thereafter, the tissues specimens were dehydrated by graded alcohol solutions (in 75%, 80%, 95%, 100% ethanol), washed with xylene as a clearing agent and incubated in the paraplast embedding medium (Sigma, Deisenhofen, Germany). Following incubation, paraplast-embedded specimens were cut into 5-6 µm thick serial sections and floated on a water bath containing distilled water heated to approximately 42°C. The selected sections were picked up with the precoated slides and dried horizontally on a warming tray overnight at 37°C. Paraplast-embedded ileal specimens were dewaxed in xylene, hydrated in graded alcohol solutions (in 100%, 100%, 95%, 80%, 75% ethanol) and immersed in distilled water. Endogenous peroxidase was blocked with 3% aqueous hydrogen peroxide solution for 30 minutes at room temperature, and nonspecific binding was blocked by 5% rabbit serum and 5% pig serum diluted in phosphate-buffered saline (PBS), for 30 minutes prior to staining. Then, the sections were incubated overnight at 4°C with mouse mAb against cytokeratin peptide 18 (1:50 dilution). The secondary antibody F(ab')<sub>2</sub> rabbit anti-mouse IgG HRP conjugate diluted 1:500 in PBS was incubated at room temperature for 1 hour. The reaction was visualized using a 0.05% solution of 3,3'-diaminobenzidine tetrachloride (DAB) in 0.05 M Tris-HCl (pH 7.6) containing 0.01% H<sub>2</sub>O<sub>2</sub>.

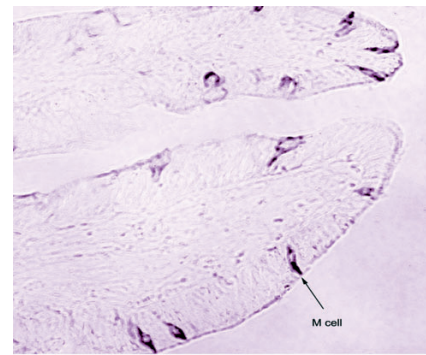
The slides were dehydrated by graded alcohol solutions and mounted in Canada balsam. The number of M cells in immunohistochemically stained tissue sections of ileum was determined by the computer-assisted quantitative analysis. A light microscope equipped with a video camera was interfaced with a desktop computer containing live video/computer graphics adapter and commercial imaging Lucia G software.

Statistical validation was performed on 12 randomly selected digital image fields of the average area of 700,480 µm<sup>2</sup> per 5 tissue sections of 2 pigs from each of three experimental groups. The antecedent testing showed that, analyzing more than 10 fields in each of 5 tissue samples from one pig per group, there were no significant deviations in the cell counts of individual pig. Levels of significance of differences between principal and control pigs were determined by two-tailed Student's *t*-test. The results deviating from the null hypothesis at a level of P<0.01 were considered as significant.

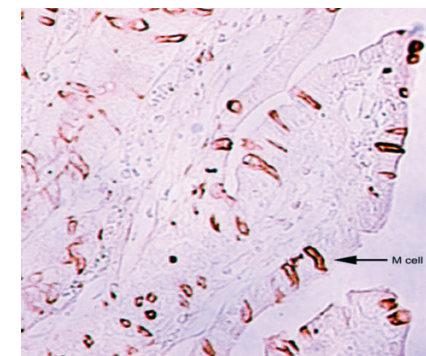
## Results and Discussion

Figures 1, 2 and 3 show the immunohistochemical identification of cytokeratin peptide 18<sup>+</sup> M cells within the villous epithelium of the ileum of weaned pigs. It is visible that these cells were quite scarce in the ileum of control/nonpretreated and challenged pig (Figure 1). Conversely, numerous M cells were found within the villous epithelium of ileum of levamisole-primed and challenged pig (Figure 2). As in the control/nonpretreated and challenged pigs, the M cells were rarely and diffusely scattered as well in the villous epithelium of ileum of vaccinated and challenged pigs (Figure 3). Generally, the M cells are rather solitary interspersed between enterocytes than as in small clusters (Figures 1, 2 and 3), and none were found to be located within the FAE regions of the ileum of 6-weeks-old pigs. The question of how does and where these cells are distributed in the immunologically mature pigs (older than 7-9 weeks) remains still unanswered.

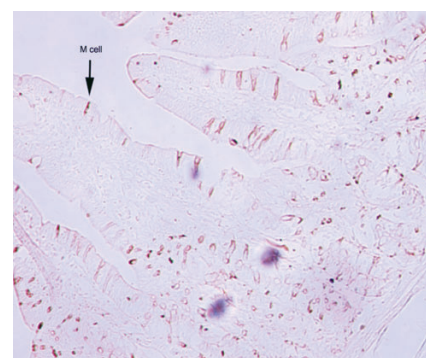
Numerical data on ileal M cells of pigs from three experimental groups as determined by computer-assisted morphometric analyses are shown in Table 1. In addition, the proportions of M cells were expressed as a ratio between number of M cells in the principal groups (no. increased or decreased) and that in the control group (where no. of the cells = 100% or 1.00). The quantitative phenotypic analyses showed that levamisole-primed and challenged pigs had significantly increased numbers (P<0.01) of ileal M cells, compared with the values obtained in the control nonprimed and chal-



**Figure 1.** Immunohistochemical finding of cytokeratin peptide 18<sup>+</sup> M cells in ileal villous epithelium of control (at day 0, *i.m.* received saline as a placebo) and challenged (at day 7, perorally received pathogenic F4ac<sup>+</sup> EPEC strain) pig aging 6 weeks; original magnification 400x.



**Figure 2.** Immunohistochemical finding of cytokeratin peptide 18<sup>+</sup> M cells in ileal villous epithelium of levamisole-primed (at days -2,-1 and 0, *i.m.* received the drug) and challenged (at day 7, perorally received pathogenic F4ac<sup>+</sup> EPEC strain) pig aging 6 weeks; original magnification 400x.



**Figure 3.** Immunohistochemical finding of cytokeratin peptide 18<sup>+</sup> M cells in ileal villous epithelium of experimentally vaccinated (at day 0, perorally received vaccine candidate F4ac<sup>+</sup> non-EPEC strain) and challenged (at day 7, perorally received pathogenic F4ac<sup>+</sup> EPEC strain) pig aging 6 weeks; original magnification 400x.

lenged pigs. The proportion of these cells in levamisole-pretreated pigs was 76% higher. In the vaccinated and challenged pigs only slightly increased (by 6%) proportion of M cells was recorded. However, this increase was not significantly different from the numerical values obtained for control pigs.

Recently, we have reported, based on the histomorphometric quantification of porcine intestinal immune cells, that it is possible to differentiate the responses of pigs immunized with levamisole-adjuvanted experimental mucosal vaccines against PWC from those of nonimmunized pigs.<sup>10,11</sup> Based upon these observations, we highlighted the query of whether and how the already recognized immunogenicity/adjuvanticity of levamisole<sup>8,9</sup> can influence *de novo* differentiation of the antigen-sampling M cells and consequently provide more feasible and effective immunization with mucosally delivered vaccines against enteric pathogens, such as porcine ETEC. In the case of positive answer, the study on development of an effective M cell-targeted vaccine against porcine PWC should be adjuvanted with levamisole in order to circumvent weak or tolerogenic responses. The successful protection of pigs vaccinated against PWC established by levamisole-adjuvanted live avirulent *E. coli* vaccine was previously reported.<sup>17</sup> Moreover, these authors showed that pretreatment of weaned pigs with levamisole synergizes the effectiveness of a live oral F4ac<sup>+</sup> non-ETEC vaccine by stimulating T cell responses in the ileal Peyer's patches.<sup>8</sup> More recent research has shown that levamisole may also elicit intestinal humoral immune response of weaned pigs to vaccine candidate F4ac<sup>+</sup> non-ETEC strain by stimulating proliferation of intestinal IgA<sup>+</sup> plasma cells.<sup>9</sup>

The present study confirmed cytokeratin 18 peptide as a specific marker for porcine M cells, which enabled their identification, localization and morphometric quantification in the ileal mucosa of weaned pigs. The cytokeratin 18<sup>+</sup> cells were found only within villous epithelial layer, regardless the treatments applied. Up to our knowledge, this is the first report on such location of M cells in swine. This finding is in agreement with the recent identification and characterization of the intestinal villous M cells in rodents and humans.<sup>18</sup> These authors postulated that villous M cells might develop from the immature enterocytes upon exposure to foreign antigens or pathogens. Since the pigs in our experiment were conventionally reared, and additionally exposed to the antigens of non-ETEC/ETEC strains, it is very unlikely that they had none of traditional FAE-associated M cells. However, we did not observe M cells within the FAE region of the ileum. Ileal villous M cells were significantly numerous ( $P < 0.01$ ) in levamisole

**Table 1. Morphometric values of cytokeratin peptide 18<sup>+</sup> M cells in villous epithelium of the ileum from pigs immunized either with levamisole (day -2, -1 and 0) or with vaccine candidate F4ac<sup>+</sup> non-ETEC strain (day 0) and challenged with F4ac<sup>+</sup> ETEC strain (day 7); the results are expressed as mean values and standard deviations (M±SD) of number of the cells per μm<sup>2</sup> of tissue section field.**

Treatment <sup>a</sup>	M±SD number of ileal M cells in 6-weeks-old pigs <sup>b</sup>	Index <sup>c</sup>	Increase/decrease
None <sup>d</sup> + F4ac <sup>+</sup> ETEC	6.12×10 <sup>-5</sup> ±9.44×10 <sup>-6</sup>	1.00	/
Levamisole + F4ac <sup>+</sup> ETEC	1.08×10 <sup>-4</sup> ±1.25×10 <sup>-5*</sup>	1.76	+0.76
F4ac <sup>+</sup> non-ETEC + F4ac <sup>+</sup> ETEC	6.47×10 <sup>-5</sup> ±1.95×10 <sup>-6</sup>	1.06	+0.06

<sup>a</sup>Groups comprised five 4-weeks-old pigs each. <sup>b</sup>As counted in 12 randomly selected fields of the average area of 700,480 μm<sup>2</sup> per sample from 2 pigs (euthanized at day 13) per group. <sup>c</sup>Ratio between number of M cells in the principal groups and that (no. of the cells = 100% or 1.00) in the control group. <sup>d</sup>Control pigs received saline at day 0 as a placebo. \*Significantly higher ( $P < 0.01$ ) than in the control pigs.

pretreated/challenged pigs, and only slightly, but not significantly numerous, in vaccinated/challenged pigs compared with nonpretreated/challenged control pigs. The latter observation could be explained by our previous experiment, where vaccine candidate F18ac<sup>+</sup> non-ETEC strain was passing through the intestines almost three times faster than F4ac<sup>+</sup> ETEC challenge strain.<sup>10</sup> Unlike F4ac<sup>+</sup> ETEC challenge strain used in the latter and current study, it seems that both vaccine candidate strains (F4ac<sup>+</sup> and F18ac<sup>+</sup> non-ETEC) exhibited weaker affinity for adhesion to the enterocyte receptors. Such adhesion is a prerequisite for stimulation of the intestinal mucosal immunity preceded by development of M cells from enterocytes upon exposure to enteric pathogens, *i.e.* F4ac<sup>+</sup> ETEC.

The origin and differentiation mechanism of M cells are still matter of controversy. It has been postulated that M cells represent a separate cell lineage that does not derive from enterocytes in the dome regions.<sup>19</sup> These authors imply that bacteria-induced up-regulation of particle transport into Peyer's patch domes can be ascribed to an increased transportation rate of M cells, but not to an increased *de novo* formation of M cells. On the other hand, several authors suggested that transport of intraluminal antigens can be up-regulated by *de novo* formation of M cells from enterocytes.<sup>20,21</sup> Recently, it has been identified a possible intermediate M cell/enterocyte cell type in upper regions of the dome in pigs.<sup>22</sup> Since it is recognized that lymphoepithelial interactions and soluble factors provide important signals for differentiation of M cells,<sup>23</sup> it would be reasonable to assume that levamisole might indirectly influence *de novo* formation of porcine intestinal villous M cell phenotype by stimulating differentiation and proliferation of lymphocyte subsets in the lamina propria and Peyer's patches of weaned pigs, as we reported earlier.<sup>8,10,11,17,24</sup>

The current study has implied that levamisole may also affect differentiation and proliferation of porcine M cells by increasing

their numbers in the intestinal villous epithelium. Historically, nearly all attention was focused on M cells within the FAE and their interactions with the underlying immune cells residing Peyer's patches, the major inductive sites in the intestine. However, current evidence indicates that the intestinal villous M cells are functionally analogous to the FAE-residing M cells and that they may compensate for their functions, independently of Peyer's patches. Thus, it would be tempting to suggest that our observation of levamisole-stimulated proliferation of the ileal villous M cells may have some relevance to the process of delivery of new mucosal vaccines against porcine PWC to the immune cells populating intestinal lamina propria. However, the interactions between M cells lying on the intestinal villi and the immune cells distributed in the underlying lamina propria are still largely unknown and further studies are required.

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