

## Functional and phenotypic analyses of porcine gut immune cells immunized by oral administration of F4ac<sup>+</sup> nonenterotoxigenic *Escherichia coli* strains

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**ABSTRACT:** The aim of this study was to determine the priming effect of experimentally inoculated non-ETEC strains (2407, 1466) on gastrointestinal mucosal lymphocytes. Five 4-week-old pigs per group were orally inoculated with either F4ac<sup>+</sup> (1466 or 2407) or F4<sup>-</sup> (1467) non-ETEC strains. The control pigs were given broth containing 1.2% sodium bicarbonate. At postinoculation Day 6 the pigs were killed, their gut lymphocytes were isolated, and their responsiveness was tested *in vitro* with F4 antigen, peptidoglycan monomer (PGM), pokeweed mitogen (PWM), phytohemagglutinin (PHA) and lipopolysaccharide (LPS). Additionally, the patterns of cluster of differentiation (CD) antigen expression on T and B cells in the single cell suspensions from JLP, IPP, and MLN were determined by flow cytometry using anti-swine CD-specific monoclonal antibodies. F4ac<sup>+</sup> non-ETEC strain 2407 and, to a lesser extent 1466, activated lymphocytes from PP and MLN to respond better to common mitogens (PHA, PWM, LPS), purified fimbrial (F4ac) antigen or immunologic response modifier (PGM). An increase of CD2a<sup>+</sup> and CD8a<sup>+</sup> T cells in JLP, and species-specific SWC1<sup>+</sup> T cells in MLN ( $P < 0.05$ ) was detected in 2407-treated pigs. In conclusion, inoculation with non-ETEC strain 2407 exhibited stimulatory properties to porcine gut immune cells, and thus, could be used in the vaccination programs to control the postweaning colibacillosis in pigs.

**Keywords:** postweaning colibacillosis; lymphocyte; pigs

Postweaning colibacillosis caused by enterotoxigenic *Escherichia coli* (ETEC) strains is an increasing problem in high-herd-health units (Winkelman, 1995). ETEC – induced pathogenesis depends on its ability to colonize small intestines and to produce enterotoxins. Adhesion of ETEC to enterocytes is accomplished by fimbrial adhesins (Nagy and Fekete, 1999). According to study conducted by Alexa *et al.* (2001), *E. coli* strains bearing F4 (K88) fimbrial adhesin prevailed among those isolated from post-weaning diarrhea. Age and genetic background seem to determine the inherent susceptibility of pigs to *E. coli*. Resistance is achieved by failure to produce the receptor on epithelial brush border membranes to which fim-

brae adhere (Francis *et al.*, 1998). The facts that F4 fimbriae are proteinaceous and found on the outside of ETEC had raised considerable interest in studies aimed to develop fimbriae – targeted vaccine against swine colibacillosis (Francis and Willgoos, 1991; Van den Broeck *et al.*, 1999). However, no safe and effective vaccine against postweaning colibacillosis is available due to unusual features of local presentation of antigens in the gut-associated lymphoid tissues (GALT), probably Peyer patches (Stokes *et al.*, 1996).

Porcine mucosal immune system is functionally immature at birth, but develops during perinatal period reaching adult values between 5 and 7 weeks of life (Bianchi *et al.*, 1992; Vega-Lopez *et*

*et al.*, 1995; Pabst and Rotkötter, 1999). It has been found that F4<sup>+</sup> antigen of ETEC strains stimulated the production of IgA and IgG antibodies (Dean-Nystrom *et al.*, 1992) and T cell mediated immune responses in the intestinal mucosa of experimentally inoculated pigs (Valpotić *et al.*, 1994). The induction of anti-ETEC IgA responses in the intestine, is dependent on T cell help and requires cognate interactions between T and B lymphocytes within GALT (Husband *et al.*, 1999). Primary contact with novel antigens takes place in the Peyer's patches and mesenteric lymph nodes. From these sites T and B lymphocytes migrate to the intestinal lamina propria. Wilson *et al.* (1996) and Haverson *et al.* (2000) have shown that the expression of major histocompatibility complex (MHC) class II antigens that are necessary for recognition of an antigen by T cells occurs on endothelial and dendritic cells in the intestinal villi. The extensive expression of MHC class II antigen on cells not normally associated with direct presentation of processed antigens (such as endothelial and epithelial cells of rats and humans) may contribute to tolerance as a result of apoptosis and clonal deletion of highly differentiated T cells (Bailey *et al.*, 1996). However, no MHC class II molecules has been found on porcine enterocytes (Haverson *et al.*, 2000).

In order to determine if a local (intestinal) immune response to fimbrial antigens of *E. coli* strains by porcine gut lymphocytes is functionally effective immediately after weaning, we have undertaken this study. Namely, either F4<sup>+</sup> or F4<sup>-</sup> non-ETEC strains were intragastrically inoculated to weaned pigs and their priming effect was assessed *in vitro* using lymphocytes from the jejunal lamina propria (JLP), ileal Peyer's patches (IPP), and the mesenteric lymph node (MLN).

## MATERIAL AND METHODS

### Pigs

Twenty 4-week-old crossbred pigs (Swedish Landrace × Yorkshire), of both sexes, weighing in average 3.9 kg, were purchased from a commercial swine farm in Croatia. They were selected at weaning from the litters of either second or third parity sows. The pigs were randomly assigned to four experimental groups comprising five animals each. They were managed, housed and fed as described (Sarmiento *et al.*, 1988).

### Inoculous strains of *E. coli*

The non-ETEC fimbriated (F4ac<sup>+</sup>) strains 2407 (O9 : K36 : H19 : K88ac, LT<sup>-</sup>, STb<sup>-</sup>) and 1466 (O9 : K36 : H19 : K88ac, LT<sup>-</sup>, STb<sup>-</sup>) as well as nonfimbriated (F4<sup>-</sup>) strain 1467 (O9 : K36 : H19 : K88ac<sup>-</sup>, LT<sup>-</sup>, STb<sup>-</sup>) were used as the inocula. Strains 1466 and 2407 were derived from strain 1467, by inserting either wild type or recombinant plasmids encoding expression of F4ac fimbrial antigen, respectively. A 60 ml of freshly growth culture containing  $1 \times 10^{10}$  viable *E. coli* per ml was given via orogastric tube to five 4-week-old pigs. The strains were grown aerobically in Trypticase soy broth (TSB) for 16 hours at 37°C. Five control animals received 60 ml of TSB with 1.2% sodium bicarbonate only.

### Sampling

All pigs were euthanized with an overdose of sodium pentobarbital at Day 6 after the inoculation. Specimens (20 cm-segments) collected from the mid portions of the jejunum and the ileum, and a whole ileal mesenteric lymph node were placed in Medium 199 (Institute of Immunology, Zagreb, Croatia).

### Isolation of gut lymphocytes

Lymphocytes from the jejunal lamina propria (JLPL) and ileal Peyer's patches (IPPL) were isolated by enzyme digestion procedure (Wilson *et al.*, 1991) using 100 U/ml or 75 U/ml of collagenase (Sigma, St. Louis, USA), respectively, and separated as the single cell suspensions by density gradient centrifugation on Lymphoprep (specific density 1.007 g/ml; Nycomed, Oslo, Norway) at  $400 \times g$  for 20 minutes. The ileal mesenteric lymph node lymphocytes (MLNL) were flushed out and washed by Medium 199 prior to centrifugation on Lymphoprep. The cells were harvested from the interface, resuspended in culture medium RPMI 1640 (Institute of Immunology, Zagreb, Croatia) supplemented with 10% of human AB serum, and their viability was determined by Trypan blue exclusion test.

### Lymphocyte stimulation test

The JLPL, IPPL, and MLNL were set-up in triplicates ( $2.5 \times 10^5$  cells per well) in 96-well round

bottom microtitre plates (Greiner, Nurtingen, Germany). The lymphocytes were stimulated with 5 µg/ml of either mitogens (phytohemagglutinin; PHA; pokeweed, PWM Wellcome, Dartford, England, or lipopolysaccharide; LPS, Sigma, St. Louis, USA), F4 specific antigen (NADC, Ames, IA, USA) or peptidoglycan monomer; PGM, Pliva, Zagreb, Croatia), and incubated at 37°C in humidified atmosphere with 5% CO<sub>2</sub> in air for 72 hours. Pulse labeling of stimulated cells was performed by adding 1 µCi per well of <sup>3</sup>H-thymidine (specific activity: 5 µCi/mM; Amersham, England) 18 hours prior to harvesting. The cells were harvested by Skatron cell harvester (Skatron, Lier, Norway), dried on Skatron GF/C filters and transferred into counting vials with 3 ml of scintillation liquid (Merck, Darmstadt, Germany). The incorporation of <sup>3</sup>H-thymidine into newly-synthesized DNA of stimulated cells was measured in counts per minute (cpm) by β-scintillation counter Delta 300 (Nuclear, Chicago, USA). The mean cpm ± SD of triplicate cultures were expressed as the stimulation index (SI) by the following calculation as follows:

$$SI = \frac{\text{mean values of cpm in stimulated cultures}}{\text{mean values of cpm in nonstimulated cultures}}$$

### Monoclonal antibodies (mAbs)

The mAbs reactive with cell surface molecules on porcine lymphocytes, i.e. cluster of differentiation (CD) antigens, such as: CD1 (anti B cell mAb 76-7-4), CD2a (anti T cell mAb MSA4), CD4a (anti T-helper cell mAb 74-12-4), CD8a (anti T-cytolytic cell mAb 76-2-11), SWC1 (anti T cell mAb 76-6-7), and SWC2 (anti T cell mAb PG124A), were kindly provided by Dr. Pescovitz (Indianapolis, IN, USA), Drs. Hammerberg/Lunney (Beltsville, MD, USA), and Dr. Davis (Pullman, WA, USA).

### Flow cytometry

The suspensions containing  $1 \times 10^6$  of freshly prepared JLPL, IPPL, or MLNL per ml were mixed with 50 µl of each mAb listed above for 30 minutes at 4°C. The cells were washed two times in phosphate buffered saline (PBS) by centrifugation, and incubated with 50 µl of rabbit anti-mouse IgG conjugated with fluorescein isothiocyanate (Institute of Immunology, Zagreb, Croatia) for 30 minutes at

4°C in the dark chamber. Polyclonal mouse serum was used as a negative control. Blocking of the nonspecific binding was performed by addition of 1% NaN<sub>3</sub> in PBS. Then the lymphocytes were washed two times with PBS and quantified in the flow cytometer EPICS (Coulter Electronics, Hialeah, USA) by counting of 50 000 cells from each sample. The percentage of mAbs-designated CD<sup>+</sup>/SWC1<sup>+</sup> lymphocyte (T and B) subsets was obtained for each lymphoid tissue tested.

### Statistics

The levels of significance were determined by the *t*-test using computer program Statgraph 4.2. (Statgraphics, Inc., Rockville, USA).

## RESULTS

Lymphocytes from the MLN ( $P < 0.01$ ) of 2407-inoculated pigs responded more strongly to stimulation with PGM, PWM, LPS and PHA than did the cells from control pigs (Figure 1). Consistent with this is better responsiveness of the lymphocytes from IPP when LPS ( $P < 0.01$ ) was applied (Figure 2). However, F4 antigen, when stimulated MLNL and IPPL ( $P < 0.01$ ) induced suppression (Figures 1 and 2).

Responses of MLNL and IPPL to PGM and PHA from 1467- and 1466-inoculated pigs were significantly higher ( $P < 0.01$ ) than that of control pigs (Figures 1 and 2) with the exception of IPPL response to PHA in 1467-inoculated pigs. Conversely, the JLPL of pigs inoculated with 1466 exhibited much lower ( $P < 0.01$ ) responses to all stimuli, except PGM (Figure 3).

Generally, porcine MLNL proliferated better to all stimuli, except to F4 antigen than IPPL, whereas proliferation of JLPL was suppressed in this model system.

In 2407-inoculated pigs CD8a<sup>+</sup> T cells from MLN, IPP and JLP were more frequent ( $P < 0.01$ ) than those from respective tissues of control pigs (Figures 4–6). Similar finding was obtained with JLPL when 1466 strain was inoculum.

Species-specific SWC1a<sup>+</sup> T cells were more abundant in MLN ( $P < 0.01$ ) of 2407-inoculated pigs (Figure 4). Also, CD4a<sup>+</sup> helper T cells were more numerous ( $P < 0.01$ ) in IPP of these pigs (Figure 5).

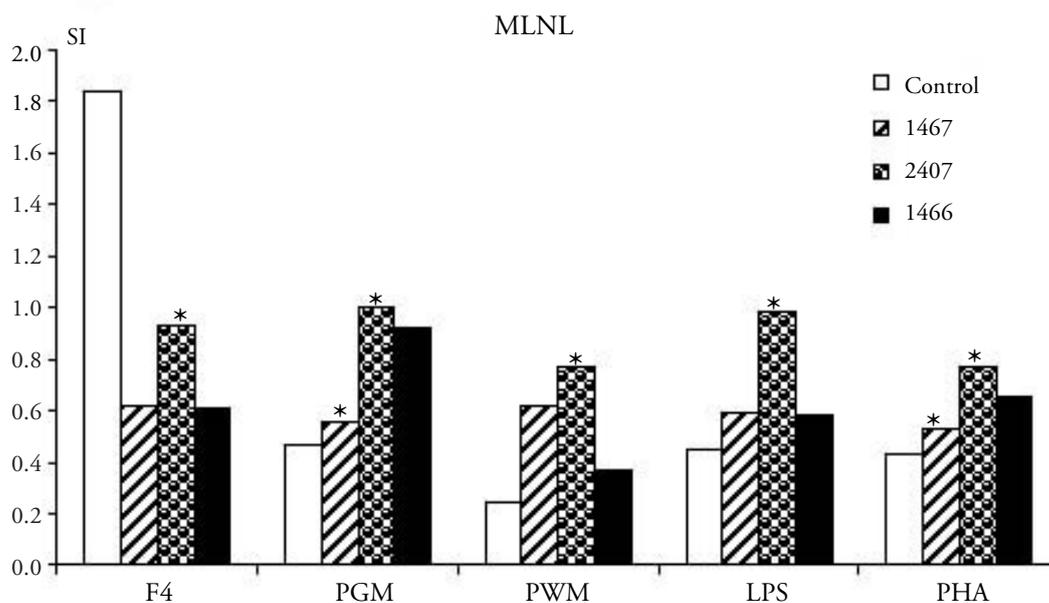


Figure 1. *In vitro* responses of mesenteric lymph node lymphocytes (MLNL) to F4, PGM, PWM, LPS, or PHA, in five 4-week old pigs per group intragastrically inoculated with either F4ac<sup>+</sup> or F4<sup>-</sup> non-EPEC strains (2407, 1466 or 1467, respectively). Control pigs received TSB only. All pigs were euthanized 6 days after the inoculation. Data are expressed as the SI values; significantly lower or higher (\*) at  $P < 0.01$  than in the control (TSB-treated) pigs

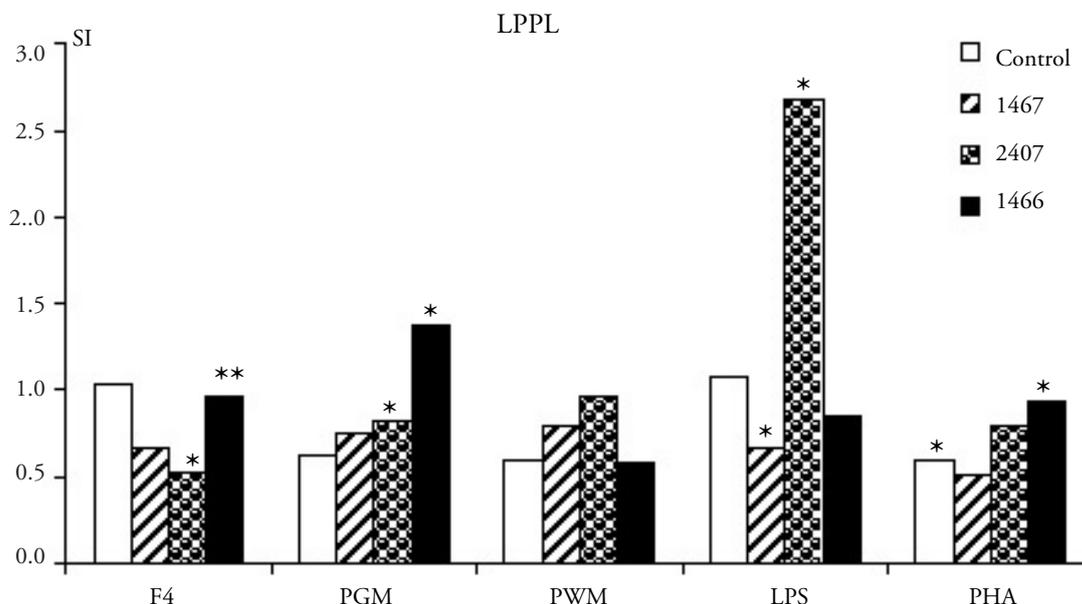


Figure 2. *In vitro* responses of ileal Peyer's patch lymphocytes (IPPL) to F4, PGM, PWM, LPS, or PHA in five 4-week-old pigs per group intragastrically inoculated with either F4ac<sup>+</sup> or F4<sup>-</sup> non-EPEC strains (2407, 1466 or 1467, respectively). Control pigs received TSB only. All pigs were euthanized 6 days after the inoculation. Data are expressed as the SI values; significantly lower or higher (\*) at  $P < 0.01$  than in the control (TSB-treated) pigs

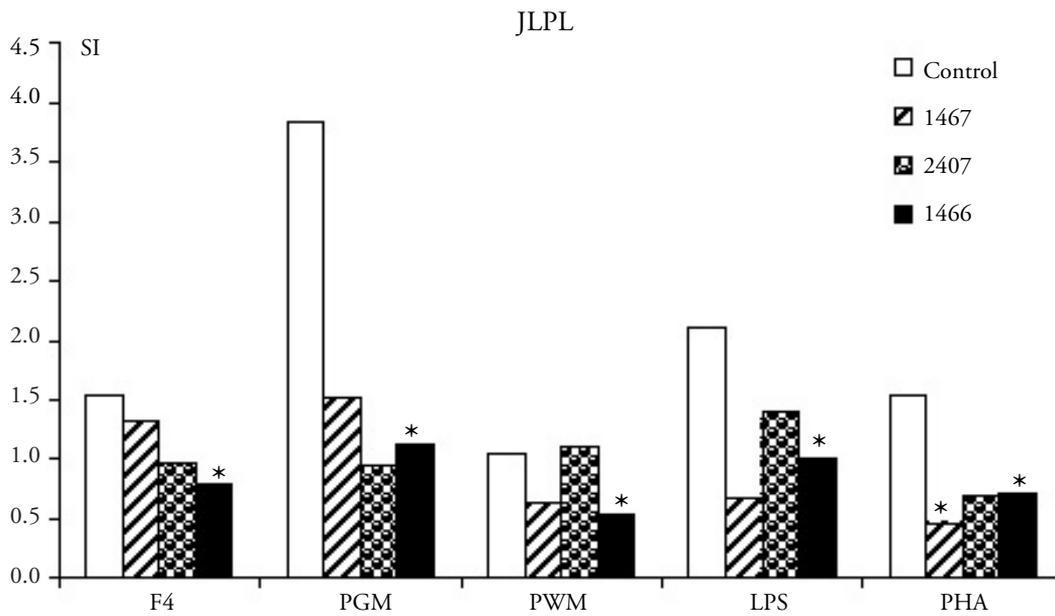


Figure 3. *In vitro* responses of jejunal lamina propria lymphocytes (JLPL) to F4, PGM, PWM, LPS or PHA in five 4-week-old pigs per group intragastrically inoculated with either F4ac<sup>+</sup> or F4<sup>-</sup> non-ETEC strains (2407, 1466 or 1467, respectively). Control pigs received TSB only. All pigs were euthanatized 6 days after the inoculation. Data are expressed as the *SI* values; significantly lower (\*) at  $P < 0.01$  than in the control (TSB-treated) pigs

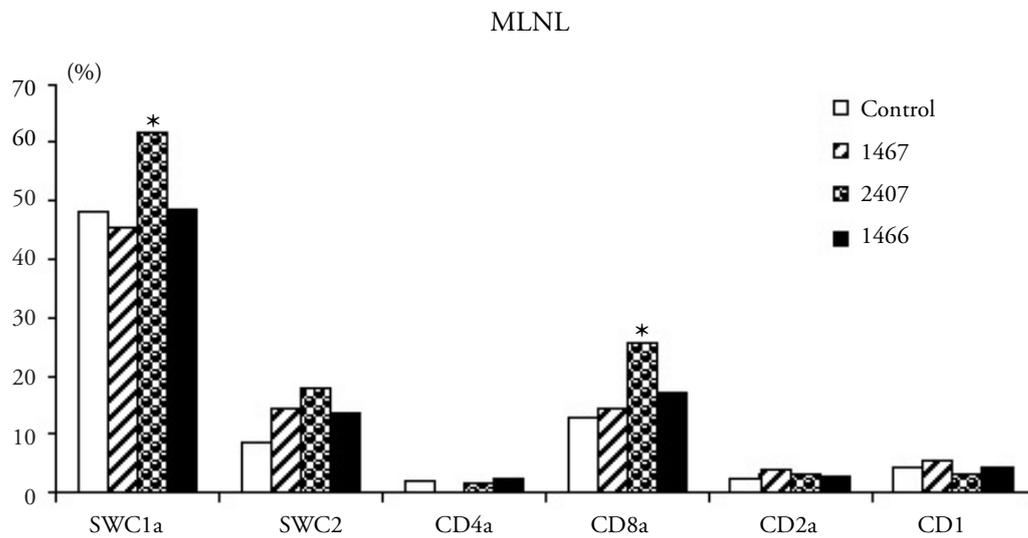


Figure 4. Flow cytometric identification and quantification of CD<sup>+</sup> T and B lymphocyte subsets in the cell suspensions from MLN in five 4-week old pigs per group intragastrically inoculated with either F4ac<sup>+</sup> or F4<sup>-</sup> non-ETEC strains (2407, 1466 or 1467, respectively). Control pigs received TSB only. All pigs were euthanatized 6 days after the inoculation. Data are expressed as the *SI* values; significantly lower or higher (\*) at  $P < 0.01$  than in the control (TSB-treated) pigs

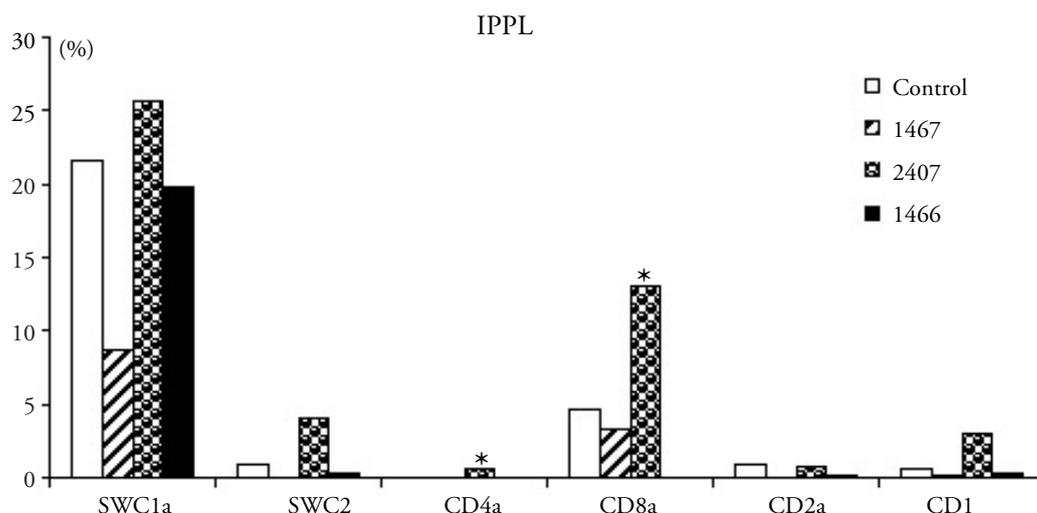


Figure 5. Flow cytometric identification and quantification of CD<sup>+</sup> T and B lymphocyte subsets in the cell suspensions from IPPL in five 4-week old pigs per group intragastrically inoculated with either F4ac<sup>+</sup> or F4<sup>-</sup> non-ETEC strains (2407, 1466 or 1467, respectively). Control pigs received TSB only. All pigs were euthanatized 6 days after the inoculation. Data are expressed as the SI values; significantly lower or higher (\*) at  $P < 0.01$  than in the control (TSB-treated) pigs

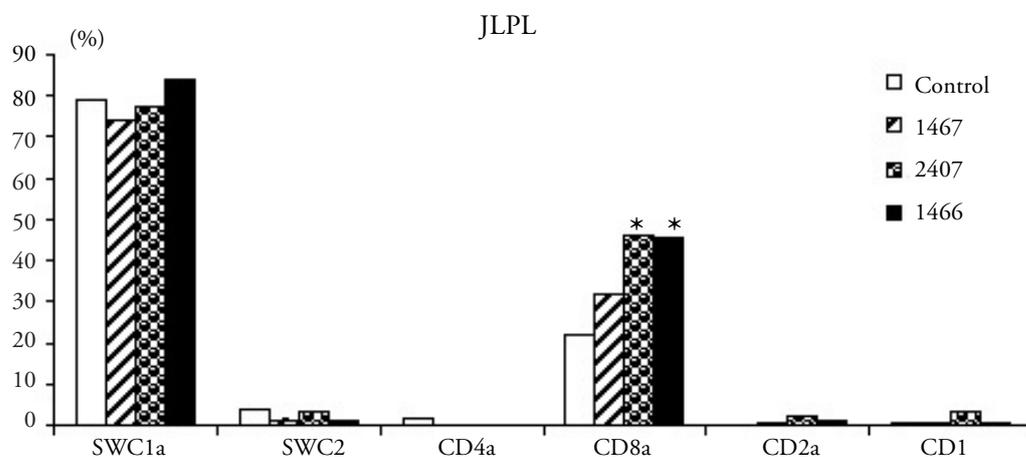


Figure 6. Flow cytometric identification and quantification of CD<sup>+</sup> T and B lymphocyte subsets in the cell suspensions from JLP in five 4-week old pigs per group intragastrically inoculated with either F4ac<sup>+</sup> or F4<sup>-</sup> non-ETEC strains (2407, 1466 or 1467, respectively). Control pigs received TSB only. All pigs were euthanatized 6 days after the inoculation. Data are expressed as the SI values; significantly lower or higher (\*) at  $P < 0.01$  than in the control (TSB-treated) pigs

## DISCUSSION

There is evidence that both vaccine candidate F4ac<sup>+</sup> non-ETEC and challenge ETEC strains stim-

ulated cellular immune responses within the GALT compartments such as JLP, IPP, and MLN (Valpotić *et al.*, 1994). Considering our previous data on immunogenicity of F4ac<sup>+</sup> ETEC strain M1823 (Šver

*et al.*, 1996) it is tentative to assume that fimbrial antigen (and toxin antigens) plays a role in priming of T and B cell responses by gut immune cells to respond stronger to PHA or LPS. However, it was reported that F4ac<sup>+</sup> ETEC strain is capable to induce histological features demonstrating the cell-mediated intestinal hypersensitivity rather than protective immunity against the enteric colibacillosis in experimentally inoculated weaned pigs (Vijtiuk *et al.*, 1995). In addition, attaching and effacing activities of porcine F4<sup>+</sup> ETEC and non-ETEC strains in the small intestine (Vijtiuk *et al.*, 2001) led to the conclusion that non-ETEC strains 2407 and 1466 could induce distinct colonization with minor to moderate microvillous damage on epithelial cells, respectively, compared to M1823, which induced severe disruption of epithelial villi. Recent findings showed that immunization with F4ac<sup>+</sup> non-ETEC strain preferentially activated CD25<sup>+</sup> and CD45RC<sup>+</sup> T cells in the spleen rather than in the MLN of challenged weaned pigs (Božić *et al.*, 2000, 2002a) may suggest the stimulation of rather systemic than local immune responses in this model system. There is, however, direct evidence suggesting that predominantly epithelial cells rather than M cells may be involved in the handling of F4 antigen (Van den Broeck *et al.*, 2000). Such inappropriate presentation/recognition of the antigen by nonprofessional antigen presenting cells, i.e. enterocytes, could contribute to clonal deletion of adjacent T helper cells within intestinal organized lymphoid tissues.

Results of current research are showing that oral inoculation with the replicating antigen, i.e. non-ETEC strains, caused priming in IPPL and MLNL, but induced a suppression in JLPL in weaned pigs. The strong proliferation of SWC1a<sup>+</sup>, and CD8a<sup>+</sup> lymphocytes in MLN of pigs has been achieved by inoculation with strain 2407. This strain induced better responsiveness of MLNL to all stimulators, with exception of F4 antigen, used *in vitro*. It is well known that intestine is capable of rapid immune responses following recognition of pathogenic organisms, but such responses frequently are associated with tissue damage and loss of function (Bailey *et al.*, 1996). There is thus an additional requirement for the immune system to maintain tolerance for harmless dietary antigens or commensal intestinal microorganisms. In contrast, the presence of the bacterial adhesion (as occurred in our model system following oral immunization with fimbriated non-ETEC strains) or enterotoxin

production and a local tissue damage (as usually occurred after infections with fimbriated ETEC strains), provides signals capable of maintaining primed CD8a<sup>+</sup> T cells as functional effectors (Bailey *et al.*, 1998). Since the CD8 molecule can be also expressed by NK cells and  $\gamma\delta$  TCR<sup>+</sup> cells, it is likely that these cells of the innate immunity, may as well participate in a local (intestinal) immune response to foreign antigens such as the fimbriae of inoculated non-ETEC strain 2407. Actually, Davis *et al.* (2001) reported that 23% of porcine CD8<sup>+</sup> cells co-express the  $\gamma\delta$  TCR.

The implications of our model are that specific immunization with F4ac – bearing *E. coli* regardless of expression of other virulence factors (such as enterotoxins) led to downregulation of T cell responsiveness when F4 fimbriae were used as recall antigen. That was particularly obvious in JLP of pigs inoculated with 1466 strain, where committed T cells are not stimulated and even suppressed by proteinaceous appendages, i.e. F4 fimbriae of *E. coli*, but also by other stimuli applied in our study. This is in agreement with observation of Bailey *et al.* (1996) who suggests that T cells clonal deletion or anergy maybe the outcome of recognition of harmless dietary antigens or commensals in the normal intestine by T cells.

In the present study 2407 strain might cause priming of gut immune cells (particularly those residing MLN and IPP) through the adherence of the fimbrial antigen to enterocytes and its subsequent presentation to T lymphocytes. In support of this, there is an evidence of that CD4a<sup>+</sup> (helper T lymphocytes) and CD8a<sup>+</sup> cells were numerous distributed in the IPP of pigs inoculated with this strain.

Accumulating evidence suggests that intestinal mucosa maybe a heavily regulated and regulating environment. All memory/effector T cells become increasingly susceptible to apoptosis and increasingly dependent on their microenvironment for survival, making them extremely good targets for regulation (Bailey *et al.*, 1998).

The potential for 2407 non-ETEC strain to be used immunoprophylactically to enhance normal response to homologous ETEC antigens at mucosal sites (such as MLN) is now an attractive target for mucosal vaccine design. Strategies to achieve both improved delivery of replicating antigen together with adjuvants such as levamisole (Božić *et al.*, 2002b) and other immune response modifiers (Šver *et al.*, 1996) are now possible.

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