CD45RA and CD45RC isoforms expression in weaned pigs vaccinated with non-enterotoxigenic F4ac$^+$ *Escherichia coli* strain against colibacillosis

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ABSTRACT: Since no effective vaccine is available for its immunoprophylaxis, porcine post-weaning diarrhoea (PWD) induced by enterotoxigenic *Escherichia coli* (ETEC) strains remains an important cause of morbidity. Live attenuated oral vaccines have been suggested to be relatively effective in preventing ETEC-induced PWD in the pigs, but the mechanisms responsible for protection have not been elucidated. In the present study we have investigated the likely impact of oral vaccination of weaned pigs with non-ETEC strain expressing F4ac antigen on CD45RA and CD45RC isoforms expression on the surface of the mesenteric lymph node (MLN) cells and on spleen cells by using one-colour flow cytometry. Additionally, to assess the activation state of T cells in the MLN and spleen of the pigs, surface expression of the lymphocyte activation marker CD25 was analysed. Sham-vaccinated weaned pigs served as controls. Our results of the quantitative phenotypic analysis of isolated lymphocytes showed that the vaccinal *E. coli* strain induced elevation of both CD25$^+$ and CD45RC$^+$ cells in the spleen, but not MLN, of the vaccinated weaned pigs. This was accompanied by decreased CD45RA expression on spleen cells, suggesting that CD45RA$^+$ spleen cells could develop CD45RC phenotype, probably as a consequence of activation in the vaccinated challenge-infected weaned pigs.

Keywords: weaned pigs; vaccination; colibacillosis; CD45 isoforms; CD25; flow cytometry

The lymphocyte common antigen, CD45, is a tyrosine-specific phosphatase which may serve as a signalling molecule involved in the regulation of T and B cell-activation (Janeway, 1992; Alexander, 2000). On porcine lymphocytes at least four CD45 isoforms (CD45RAC, CD45RA, CD45RC and CD45RO with molecular weights of 240, 226, 210 and 190 kDa, respectively), produced by the alternative splicing of exons A, B and C within a single gene, can be expressed (Zuckermann *et al.*, 1998). The alternative splicing of CD45 is regulated so that naive lymphocytes predominantly express CD45RA isoform and switch to expression of CD45RO upon activation (Meeusen, 1998). However, antiswine monoclonal antibodies (mAbs) specific to a truly memory phenotype, CD45RO, are not yet available. Moreover, CD45RA$^+$ cells have not yet been demonstrated directly to generate CD45RC phenotype as a consequence of activation in swine.

Enterotoxigenic *Escherichia coli* (ETEC) strains expressing F4 fimbrial antigens, especially F4ac variant, are the most common cause of post-weaning diarrhoea (PWD) in susceptible weaned pigs worldwide (Valpotić *et al.*, 1992; Choi and Chae, 1999; Alexa *et al.*, 2001). However, no effective vaccine for its immunoprophylaxis is available and porcine PWD remains an important cause of morbidity (Nagy and Fekete, 1999). Candidate live attenuated oral vaccines have been suggested to be relatively effective and safe in preventing ETEC-induced PWD in the pigs (Attridge *et al.*, 1988; Francis and Willgohs, 1991; Vijtiuk *et al.*, 1995), but the mechanisms responsible for protection have not been elucidated. Although the IgA-mediated immune exclusion may provide the first acquired barrier against a variety of mucosal infectious processes (Husband, 1993; Husband *et al.*, 1999), a body of evidence also suggests that both vaccinal non-ETEC and pathogenic

ETEC strains expressing F4ac fimbrial antigen may stimulate the generation of T cell-mediated immunity in weaned pigs (Valpotić *et al.*, 1994). Surprisingly, we have recently found that vaccination with F4ac^{+} non-ETEC strain did not activate intestinal CD25^{+} T cells in challenge-infected weaned pigs (Božić *et al.*, 2000). It also failed to efficiently stimulate recruitment of CD45RC^{+}, but not of CD45RA^{+}, cells in the gut mucosa of these pigs, and this was correlated with low protection of the pigs from challenge-induced clinical disease (Božić *et al.*, 2001).

The expression of restricted epitopes of CD45 molecule (CD45R) by mesenteric lymph node (MLN) or spleen cells has not been investigated in weaned pigs vaccinated with a live attenuated oral vaccine against porcine PWD. Additionally, although Lacković *et al.* (1997) proposed that the presentation of bacterial antigens to the MLN T cells in weaned pigs immunised with F4ac[+] non-ETEC strain might be accompanied by upregulation of activation molecules on their surface, these authors did not analyse the expression of CD25 activation antigen. Interestingly, when isolated from weaned pigs primed with F4ac[+] ETEC, MLN and spleen T cells proliferate much more vigorously than do lamina propria (LP) or Peyer's patches (PP) cells, upon in vitro stimulation with fimbrial F4ac antigen or common mitogens (Valpotić and Stokes, 1994). Thus, the role of MLN and spleen T cells in protective immunity against porcine ETEC-infection appears more extensive than previously recognised. The aim of the present study was to evaluate CD45RA and CD45RC isoforms expression in the MLN and spleen of weaned pigs vaccinated with F4ac[+] non-ETEC strain against porcine PWD. In order to assess the activation state of lymphocytes in the MLN and spleen of the pigs, surface expression of the lymphocyte activation marker CD25 was also analysed.

MATERIAL AND METHODS

Twelve commercial crossbred (Swedish Landrace × Yorkshire) pigs weaned at 4 weeks were purchased from a herd previously shown to be susceptible to adhesion by F4ac[+] ETEC strain (Valpotić *et al.*, 1992). Pigs were randomly divided into 2 groups of 6 pigs each housed in a facility at the Veterinary Faculty of University of Zagreb, on flat-decks in isolation pens at 22 ± 2°C. Pigs were managed as detailed elsewhere (Sarmiento *et al.*, 1988). Pigs received a commercial weaner diet and had an unlimited access to water.

The vaccinal non-ETEC strain 2407 (serotype O9: K36: H19: F4ac: LT[-] STb[-]) and the challenge F4ac[+] ETEC strain 11-800/94 (serotype O149: K91: F4ac: 987P: Hly[+] LT[+] STb[+]) were used for vaccination and challenge infection, respectively. The vaccinal non-ETEC strain 2407 is a 1467 strain containing a recombinant plasmid pMK005 (Kehoe *et al.*, 1981) encoding F4ac fimbrial antigen and was obtained from Dr. T. A. Casey, NADC, Ames, IA, USA. The strain was constructed by electroportation of 1467 with pMK005 and selected for recipients with ampicillin. The presence of F4 recombinant plasmid was confirmed by slide agglutination for the expression of F4ac fimbrial antigen using anti-F4ac serum raised in rabbit, and by DNA hybridisation (both colony and southern blotting) with specific probe for F4ac (Casey and Moon, 1990). The challenge F4ac[+] ETEC strain 11-800/94 with authentic F4ac plasmid was isolated from diarrhoeic pigs reared on swine farms in Croatia (Bilić and Žutić, 1985) and was provided by Dr. V. Bilić, Department of Bacteriology, Croatian Veterinary Institute, Zagreb.

An experimental group (*n* = 6) was intragastrically vaccinated on day 0 with 10[10] colony forming units per ml (CFU/ml) of F4ac[+] non-ETEC strain 2407 in 60 ml of Trypticase soy broth (TSB), as described elsewhere (Sarmiento *et al.*, 1988). Sham-vaccinated control group (*n* = 6), housed separately, was administered TSB only. On post-vaccination day 7 each of all pigs, from both experimental and control groups, was challenge-inoculated with 10[10] CFU/ml of F4ac[+] ETEC isolate in 60 ml of TSB using an orogastric tube.

After euthanasia of pigs by T61[®] (Hoechst, München, Germany) on post-challenge day 7, the spleens and MLN were aseptically removed for isolation of splenocytes and MLN lymphocytes. Immediately after excision, the collected spleens and MLN were placed into modified Eagle's medium with Hanks (MEM-H; Institute of Immunology, Zagreb, Croatia). Lymphocytes from spleen and MLN were isolated by perfusing and teasing tissue in MEM-H medium followed by density separation of mononuclear cells over Lymphoprep (Nycomed, Oslo, Norway) and centrifugation at 750 × g for 25 min. The cells were then collected and washed once with MEM-H medium. The purified cells (>95% pure) were more than 98% viable as determined by propidium iodide staining.

The mAbs STH267 and MIL5 specific to swine CD45RA and CD45RC isoforms, respectively (Zuckermann *et al.*, 1998), tested for the Second Swine CD (cluster of differentiation) workshop held in 1995, Davis, CA, USA, and the standard mAb K231.3B2

recognising α chain of the porcine interleukin-2 receptor (IL-2R; CD25) (Bailey *et al.*, 1992) defined in the First International Swine CD workshop held in 1992, Budapest, Hungary, were used in the present study. Antibody to CD45RA (clone STH267) was donated by Dr M. Shimizu (National Institute for Animal Health, Ibaraki, Japan). The anti-CD45RC mAb MIL5 and anti-CD25 mAb K231.3B2 were provided by Prof. C.R. Stokes (University of Bristol, Bristol, UK).

Single cell suspensions were prepared and incubated with mAbs ($50\,\mu l/10^6$ cells) used in a single-colour flow cytometry to determine the percent of positive staining. The fluorescence of the mAbs labelled cells was analysed by using an EPICS C flow cytometer (Coulter Electronics, Hialeah, FL, USA). For visualisation in this electronically programmable individual cell sorter, anti-mouse fluorescein isothiocyanate (FITC)-conjugated immunoglobulin (Ig) G (Institute of Immunology, Zagreb, Croatia) was used. Data were presented as the percentages of the total numbers of cells in the lymphocyte gate expressing a specific antigen ± SD. The lymphocyte gate was set according to standard light scatter properties of swine lymphocytes.

Statistical analysis was performed using the computer program Microsoft Excel Ver. 5.0, (Microsoft Corporation, USA). Levels of significance of differences between the two groups of weaned pigs were determined by two-tailed Student's *t*-test. A value of $P > 0.05$ was considered non-significant.

RESULTS

The data obtained by single-colour immunostaining used in flow cytometry for the percentages of MLN cells and splenocytes bound by the mAbs STH267 and MIL5 which positively identified CD45RA and CD45RC cells, respectively, are depicted in Figure 1. Although a slight increase of the CD45RC$^+$ cells was observed in the vaccinated vs. sham-vaccinated challenge-infected weaned pigs, both groups contained a comparable percentage of CD45RA$^+$ (Figure 1A) and CD45RC$^+$ MLN cells (Figure 1B), there being no significant difference in cells from vaccinated, or sham-vaccinated, challenge-infected pigs. The expression of CD45RA and CD45RC isoforms on the surface of isolated spleen cells however differed between the two groups of the pigs. Significant increase ($P \leq 0.01$) of the mean percent of CD45RC$^+$ cells (Figure 1B) and marked but not statistically significant decrease of the mean percent of CD45RA$^+$ cells (Figure 1A) was observed in spleen of the vaccinated challenge-infected weaned pigs vs. sham-vaccinated infected controls.

As it can be seen from the data depicted in Figure 2, the CD45RA/CD45RC ratio was approximately similar and lower than 1 in the MLN of both vaccinated and sham-vaccinated challenge-infected weaned pigs, respectively. However, although sham-vaccinated infected controls had values in the spleen higher than in the MLN, the CD45RA/CD45RC ratio observed in
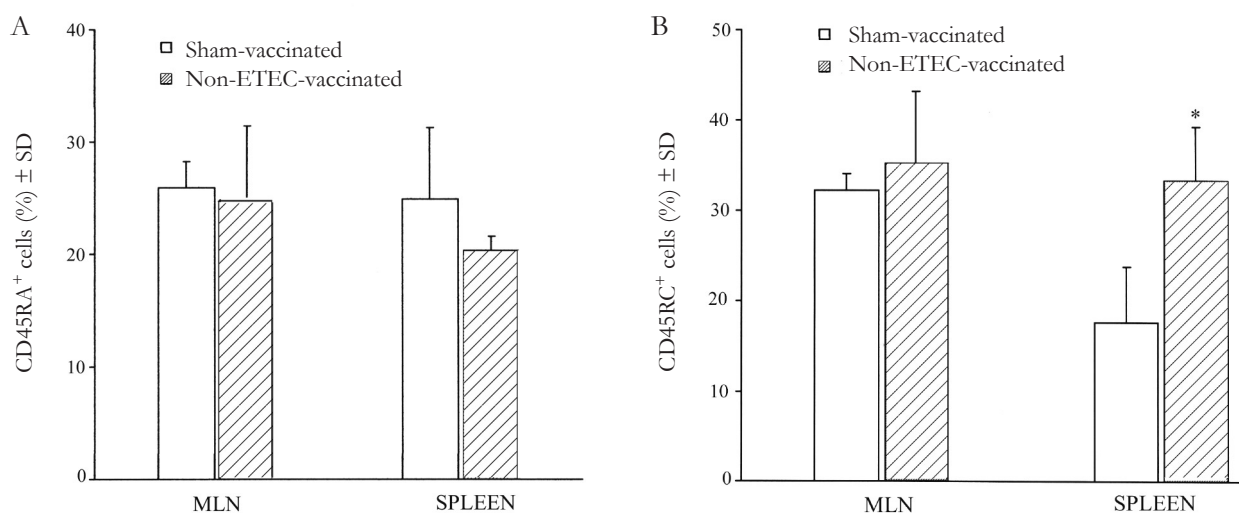

Figure 1. Flow cytometric analysis of the positively stained cells expressing CD45RA (A) and CD45RC (B) isoforms in the MLN and spleen of the vaccinated and sham-vaccinated weaned pigs challenge-infected with F4ac$^+$ ETEC strain. Experimental group ($n = 6$) was orally vaccinated on day 0 with F4ac$^+$ non-ETEC strain and the control group ($n = 6$) received TSB only. All pigs were challenge-inoculated on post-vaccination day 7 and sacrificed 7 days later

* significant difference ($P \leq 0.01$) between the two groups
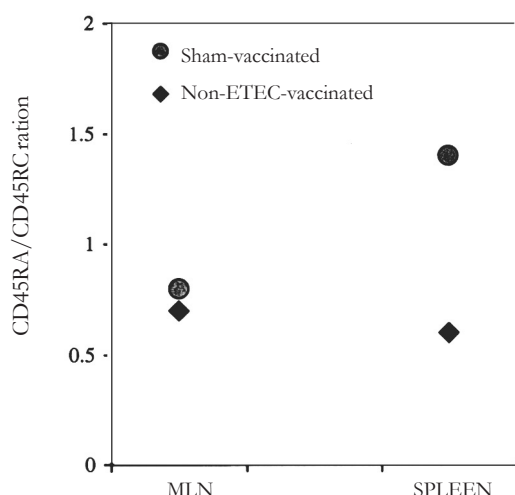
Figure 2. CD45RA/CD45RC ratio within the MLN and spleen of weaned pigs orally vaccinated (day 0) with F4ac+ non-ETEC strain (n = 6), or sham-vaccinated (n = 6), challenge-infected with F4ac+ ETEC strain on post-vaccination day 7. Pigs were sacrificed 7 days after challenge-inoculation. The ratio calculated from mean percentages of positively stained cells is plotted
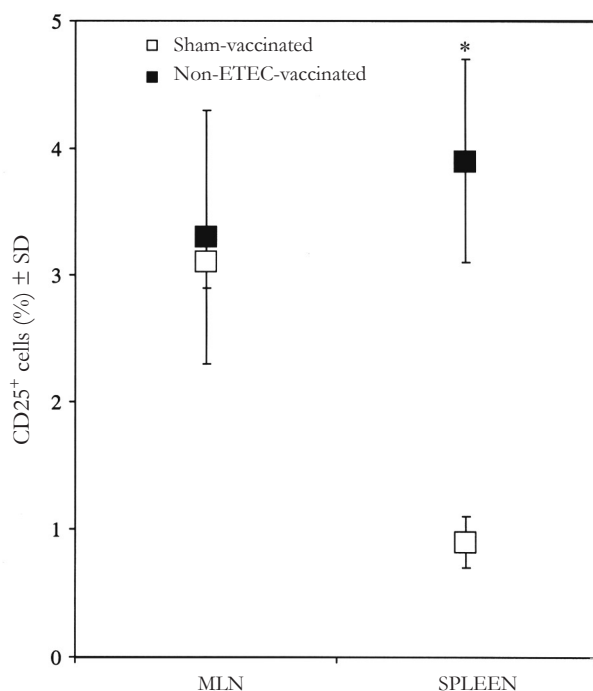

Figure 3. CD25+ cells arise in the spleen of weaned pigs (n = 6) vaccinated (day 0) with F4ac+ non-ETEC strain and challenge-infected with F4ac+ ETEC strain 7 days later, vs. sham-vaccinated infected controls (n = 6). Pigs were sacrificed 7 days following challenge-inoculation

*significant difference ($P \leq 0.005$) between the two groups

the spleen of non-ETEC-vaccinated challenge-infected weaned pigs was again lower than one.

In order to assess the activation state of lymphocytes in the MLN and spleen of the two groups of pigs, surface expression of the lymphocyte activation marker CD25 was analysed. The results from Figure 3 show that the proportion of CD25+ cells was relatively low (less than 5% of cells were positively stained) in the MLN and spleen of both vaccinated and sham-vaccinated challenge-infected groups of weaned pigs. However, although there were no variations in the proportion of CD25+ cells in the MLN between the two groups, the proportion of CD25+ spleen cells increased more than 4 times ($P \leq 0.005$) in the non-ETEC-vaccinated challenge-infected group of weaned pigs vs. sham-vaccinated infected controls.

DISCUSSION

The present study carried out on MLN cells and splenocytes in experimentally vaccinated or sham-vaccinated ETEC-infected weaned pigs reveals dichotomy in MLN and spleen cells responses induced by vaccination. No significant differences were observed between the two groups of weaned pigs either in CD45RA+ or in CD45RC+ cells within the MLN, suggesting that the vaccinal strain of *E. coli* might not be enough immunogenic for these cells in weaned pigs, as it was expected to be. By contrast, in the spleen of vaccinated vs. sham-vaccinated challenge-infected weaned pigs vaccination was found to influence significantly CD45RA and CD45RC isoforms expression in that more CD45RC+ and fewer CD45RA+ spleen cells can mean that CD45RA+ cells developed CD45RC phenotype. Our finding of CD45RA/CD45RC ratio being less than 1 in the spleen of vaccinated, but not of sham-vaccinated, challenge-infected weaned pigs corroborated this. Since CD45RA+ cells have not yet been proven directly to generate CD45RC phenotype as a consequence of activation in swine, it is tempting to speculate that vaccination with the vaccinal strain of *E. coli* stimulates a conversion of CD45RA to CD45RC isoform in the spleen, but not MLN. Consistent with these results, there is a possibility of activating CD45R+ cells in the spleen of vaccinated pigs by the vaccinal *E. coli* strain, and of promoting their expansion ultimately leading to the generation of memory cells. Indeed, the alternative splicing of CD45 is regulated so that naive leukocytes predominantly express CD45RA isoform and switch to expression of recently activated/memory phenotype upon activation

(Janeway, 1992; Meeusen, 1998). In this context it is important to note that since the CD45RA/CD45RC ratio was found to be less than 1 in the MLN of both vaccinated and sham-vaccinated challenge-infected pigs, there is also a possibility of activating CD45R$^+$ MLN cells, but irrespective of the specific vaccination.

The above data are in line with our observation of CD25 activation antigen expression on the cell surfaces. The current study shows that the exposure of pigs to the F4ac$^+$ non-ETEC vaccinal strain did not stimulate CD25 molecule expression on the surface of MLN cells in weaned pigs challenge-infected with ETEC above the level detected in sham-vaccinated infected controls. Nevertheless, these findings are suggestive of MLN T cells activation, since resting normal cells do not express the CD25 but are activated to express it after mitogen or antigen stimulation (Bailey *et al.*, 1996). In the present study, the vaccination of pigs with the vaccinal *E. coli* strain appeared to be indeed effective in sustained stimulation of CD25 expression, despite its relatively low intensity of expression. However, undiscriminating expression of CD25 by MLN cells in vaccinated and sham-vaccinated challenge-infected weaned pigs argues against the beneficial effect of vaccination on fimbrial antigen-specific MLN T cell activation. Conversely, increased CD25 expression observed on T cells in the spleen of vaccinated vs. sham-vaccinated challenge-infected weaned pigs indicates enhanced T cell-mediated immunity in this peripheral lymphoid organ induced by the action of non-ETEC strain expressing F4ac fimbrial antigen. Inasmuch as a specific vaccination of pigs with the vaccinal strain of *E. coli* preferentially activates spleen, rather than the MLN (this report) or LP T cells (Božić *et al.*, 2000), one might expect F4ac$^+$ non-ETEC strain as a truly potent systemic immunogen. Yet, oral immunisation of weaned pigs by live avirulent F4ac$^+$ *E. coli* strain elicited B cell recruitment in the LP (Lacković *et al.*, 1997), implying that this vaccinal strain may also be considered as mucosal immunogen. However, our overall immunological findings were correlated with a low protection of the vaccinated pigs from a clinical disease induced by challenge-infection (Francis and Willgohs, 1991; Božić *et al.*, 2001), suggesting that unsuccessful protection might be due to the stimulation of non-protective systemic, instead of protective mucosal T cell-mediated immune response.

It was somehow surprising that the vaccinal *E. coli* strain failed to further stimulate MLN T cells in the challenge-infected weaned pigs, since the presentation of antigen to porcine naive T cells in the gut mucosa generally occurs within the organised structures of gut-associated lymphoid tissues (GALT), ultimately leading to T cell activation and proliferation within the MLN (Stokes *et al.*, 1996; Makala *et al.*, 1998). This could be explained by preliminary results suggesting that the absortive enterocytes, but not microfold cells of the follicle-associated dome epithelia, may be involved in the binding of F4 antigens of *E. coli* in swine (Van den Broeck *et al.*, 2000). If true, then non-ETEC-derived fimbrial antigens could indeed by-pass the PP, one of the primary areas in the GALT where specific immune responses are generated (Stokes *et al.*, 1996; Makala *et al.*, 1998), subsequently resulting in the induction of non-specific systemic immune response. In this context, it is important to note that not all weaned pigs have receptors for the F4ac fimbrial antigen (Nagy and Fekete, 1999) and that only pigs that express the F4-specific receptors in the gut mucosa display an immune response (Van den Broeck *et al.*, 1999a). However, although F4-specific antibody-secreting cells in the MLN, could not be detected in F4 receptor negative pigs after oral F4 fimbriae administration (Van den Broeck *et al.*, 1999b), a priming of the systemic immune system did occur (Van den Broeck *et al.*, 2002). These data, together with the possible lack of F4ac-specific receptors in some (or more) pigs used in the present study, may further explain the absence of the stimulation of cell-mediated immune response in the MLN of the vaccinated weaned pigs upon virulent challenge with ETEC.

In conclusion, by the quantitative phenotypic analysis of isolated lymphocytes we have shown that oral vaccination of weaned pigs with F4ac$^+$ non-ETEC strain preferentially stimulates CD25$^+$ and CD45RC$^+$ cells in the spleen, rather than in the MLN (this report) or intestinal mucosa (Božić *et al.*, 2000; 2001). This was accompanied by the post-challenge diarrhoea developed by vaccinated pigs (Božić *et al.*, 2001), indicating that the regulatory cells in the spleen rather than specific effector cells in the MLN and intestinal mucosa might be induced by vaccination. These regulatory cells can upregulate activation markers after being triggered by the challenge-infection, but probably perform antigen-non-specific activities, although this remains to be further tested.

Acknowledgements

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