

Peanut and *Amaranthus leucocarpus* lectins discriminate between memory and naive/quiescent porcine lymphocytes

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Abstract

Lectins are relevant tools to isolate and characterize different cellular sub-populations. In this work, we used the lectins *Arachis hypogaea* (Peanut agglutinin, PNA) and *Amaranthus leucocarpus* (ALL), specific for GalB1, 3GalNAc, to characterize naive and memory lymphocytes from pigs, experimentally infected with the porcine rubulavirus (RvP). Our results showed that both lectins recognized preferentially lymphocytes with the CD4⁺CD8⁺ phenotype ($P < 0.05$). The phenotypic analysis of the cells recognized by these lectins indicated that PNA⁺ lymphocytes showed higher rate of the CD29 antigen (PNA⁺CD29^{high}) than ALL⁺ (ALL⁺CD29^{low}). The number of PNA⁺CD29^{high} lymphocytes increased after 8 weeks of experimental infection with RvP, and most of the ALL⁺CD29^{low} cells became CD29^{middle}. PNA⁺ lymphocytes isolated from infected pigs proliferated after stimulation with the RvP, whereas ALL⁺ cells did not. In vitro assays indicated that the ALL⁺ cells from previously infected pigs diminished from 7.5 ± 2 to $0.5 \pm 0.3\%$ after RvP stimulation; whereas PNA⁺ cells increased from 4 ± 1 to $42 \pm 2\%$, whereas no modification in ALL⁺ or PNA⁺ cellular population was identified in lymphocytes from naive animals after RvP stimulation. Our results suggest that the cellular distribution/organization of the O-glycosidically linked glycans on lymphocytes may correlate with biological functions, and that PNA could be a tool to isolate specifically porcine memory T cell subsets, whereas ALL

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1. Introduction

Lectins, which show specificity for O-glycosydically linked glycans (containing the structure Galβ1, 3GalNAcα1,0 Ser/Thr), have been widely used in the fractionation of thymocytes and lymphocyte subpopulations. By selective agglutination with peanut agglutinin (*Arachis hypogaea*), it is possible to purify murine cortical immature thymocytes (Reisner et al., 1976) and CD8⁺ T cells activated in vitro (Chervenak and Cohen, 1982; Verheijen et al., 1983; Taira and Nariuchi, 1988). The lectin from *Amaranthus leucocarpus* (ALL) possesses the capacity to interact with murine medullar thymocytes (Lascurain et al., 1994), and human naïve CD4⁺ lymphocytes (Lascurain et al., 1997). PNA and ALL bind with the highest affinity to the disaccharide Galβ1, 3GalNAc (Pereira et al., 1976; Zenteno et al., 1992).

Adult swine infected with RvP show increased infertility rates in gilts, stillbirths, mummified fetuses in pregnant sows, and epididymitis and orchitis in boars (Stephano, 1994; Ramírez-Mendoza et al., 1997). Moderate respiratory diseases have also been observed in adult infected pigs (Hernández et al., 1997). Phenotype analysis of peripheral blood mononuclear cells (PBMC) from RvP infected animals revealed increased number of both monocytes and total T lymphocytes early during infection, and increased CD4⁺CD8⁺ cells 4 weeks after infection (Hernández et al., 1998). In contrast to human or murine lymphocytes, it has been found that a substantial proportion (10–60%) of adult swine lymphocytes have the CD4⁺CD8⁺ phenotype (Saalmueller et al., 1987). This cellular subset has been identified in peripheral blood, spleen, lymph nodes, and tonsils of normal adult swine (Zuckermann and Gaskins, 1996). CD4⁺CD8⁺ lymphocytes exhibit properties of mature antigen experienced cells (Summerfield et al., 1996; Zuckermann and Husmann, 1996), these cells participate in the regulation of porcine memory T cells (Zuckermann, 1999; Hernández et al., 2001). Primed CD4⁺CD8⁺ T cells are able to induce specific immunoglobulin synthesis by primed B cells (Ober et al., 1998), and present cytotoxic activity (De Bruin et al., 2000).

The leukocyte common antigen, CD45, has been considered a marker of memory T cells. Resting or naïve murine CD4⁺ T cells express a high-molecular weight isoform (CD45RB) that is absent in activated lymphocytes, identified by the loss of CD45RB monoclonal antibodies staining (Bell et al., 1998; Zuckermann et al., 1998). It has recently been suggested that the CD29 is a potential marker of swine memory T cells (Zuckermann and Husmann, 1996). In this work, we examine, with the aid of PNA and ALL lectins, changes in the O-glycosylation pattern of porcine T cell surface glycoproteins during an in vivo virally induced process and explore whether these changes discriminate among naïve, effector, or memory T cells.

2. Materials and methods

2.1. Animals and viruses

Eleven York Landrace, Duroc crossbred domestic adult pigs (>7 months) were obtained from a commercial farm. Animals were certified to be free of the swine diseases: Aujeszky's disease, classical swine fever, and porcine rubulavirus. Seven pigs were experimentally infected by intranasal administration of 5 ml porcine rubulavirus (10^4 TCID₅₀ ml⁻¹) as previously described (Hernández et al., 1998). The infected and the four non-infected pigs, the latter used as controls, were placed individually in an isolation facility. The RvP virus was propagated in the pig kidney cell line PK-15 with MEM (supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin (Sigma, St Louis, MO, USA). Infected cell cultures were clarified by centrifugation at $10,000 \times g$ for 45 min at 4 °C and virus infectivity was titered in cell cultures in 96-wells culture plates (Falcon Labware, New Jersey, USA). Viral antigen consisted of supernatants of infected pig kidney (PK) cells and concentrated by ultracentrifugation at $100,000 \times g$ for 4 h, at 4 °C, filtered through 0.45 µm membranes, and stored in aliquots at -70 °C until use. Mumps virus stock was grown in Vero cells for 4 days in MEM medium supplemented with 2% fetal bovine serum and 50 µg/ml gentamycin (Sigma, St. Louis, MO, USA). Protein concentration in the supernatant of these cultures was determined by the method of Bradford (1976) and aliquots were adjusted to a concentration of 20 mg/ml. Before use, the supernatant aliquot was heat-inactivated (Hernández et al., 1998). Cell cultures without stimulation were used as negative control.

2.2. Antibodies

Mouse monoclonal antibodies (mAb) anti-CD4 (74.12.4, IgG2b), anti-CD8 (76.2.11, IgG2a) and anti-pig macrophage (78.2.11, IgG2a) were kindly provided by Dr. M.D. Pescovitz, Indiana University, Indianapolis, USA (Pescovitz et al., 1984). Fluorescein isothiocyanate (FITC) labeled rat anti-mouse IgG2b was from Serotec (Oxford, England). Phycoerythrin (PE) labeled-rat anti-mouse IgG2a from Zymed Lab Inc., San Francisco, CA, USA, and RD1-labeled mouse anti-human $\beta 1$ integrin-specific mAb 4B4 (CD29) from Coulter Sci., San Diego, CA, USA.

2.3. Lectins

A. leucocarpus lectin (ALL) and peanut agglutinin (PNA from *A. hypogaea*) were obtained from the corresponding plant seeds collected in Tulyehualco (Mexico) and purified on a column containing stroma from human erythrocytes as described previously (Hernández et al., 1999; Ortiz et al., 2000). ALL and PNA lectins were labeled with the *N*-hydroxysuccinimide ester of biotin (Pierce Chemical Co., Rockford IL, USA), at a label/protein ratio of 2:1 (Savage et al., 1992). Biotin-conjugated PNA or ALL was used at a concentration of 1 µg for 10^6 cells, and revealed with CyChrome-streptavidin (Pharmigen, San Diego CA, USA). Galactose (Gal) or *N*-acetyl-D-galactosamine (GalNAc) at 200 mM

concentration was used as specific inhibitor of PNA or ALL, respectively. PNA was labeled with Fluorescein isothiocyanate (FITC) obtained from Sigma, according to Lascurain et al. (1994).

2.4. *Cytofluorometric analysis*

Peripheral blood mononuclear cells (PBMC) were isolated from blood samples from infected and naive pigs by Ficoll–Hypaque (Pharmacia, Sweden) gradient centrifugation. PBMC were stained with anti-CD8, anti-CD4 mAb, and biotin-labeled PNA or ALL. After 20 min incubation on ice, cells were washed with PBS-A (100 mM sodium phosphate, NaCl 0.15 M, pH 7.2, 0.3% bovine serum albumin), and were made to react with FITC-anti-mouse IgG2b, PE-rat anti-mouse IgG2a, and CyChrome-streptavidin for 20 min incubation on ice. After final wash with PBS, cells were analyzed with a FACS Excalibur (Becton & Dickinson, Mountain View, CA, USA). For the staining of PNA⁺CD29⁺ and ALL⁺CD29⁺ lymphocytes, PBMC were stained with biotin-conjugated PNA or ALL for 20 min; after washing with PBS-A, CyChrome-streptavidin and RD1-labeled anti-human CD29 mAb were added, washed with PBS, and analyzed by flow cytometry.

2.5. *Isolation of antigen presenting cells*

Cell suspension of PBMC in PBS-A was stained with 100 µl of the mouse anti-pig macrophage-monoclonal antibody at 4 °C during 20 min. Cells were washed with sterile PBS-A and incubated with sheep anti-mouse immunoglobulin-coated magnetic particles, and positively separated using a magnetic sorter (MACS, Miltenyi Biotec Inc., Sunnyvale, CA, USA). The purified macrophages were >96% as assessed by FACScan.

2.6. *Sorting of porcine lymphocytes*

Eppendorf tubes containing suspensions of macrophage cell-depleted PBMC (10^7 per tube) in sterile PBS-A were incubated with biotin-PNA or -ALL. After 20 min incubation on ice, cells were washed with PBS-A and reacted with CyChrome-streptavidin. After a final wash, cells were suspended at 10^7 ml⁻¹ in PBS and sorted with the FACS Excalibur into PNA⁺ and ALL⁺ cells. Sorted cells were incubated immediately for 15 min with 0.2 M GalNAc or Gal to eliminate ALL and PNA, respectively. The phenotype of sorted cells was analyzed using anti-CD4 or -CD8 mAb and revealed with FITC-labeled rat anti-mouse IgG2b and PE labeled-rat anti-mouse IgG2a. No differences in phenotype characterization were obtained in control experiments performed in the presence of lectins. Stained unsorted and unstained cells served as controls for lymphoproliferation assays.

2.7. *Lymphoproliferation assays*

PNA⁺ and ALL⁺ electronically sorted lymphocytes from infected and naive pigs were cultured at 2.5×10^5 per well in sterile 96-wells round bottom plates in 200 µl RPMI

culture medium (HEPES-buffered RPMI-1640, Sigma) supplemented with 2×10^{-5} M 2-mercaptoethanol, 2 mM sodium pyruvate, 2 mM L-glutamine, 1 µg/ml gentamycin, and 10% fetal calf serum). Lymphocytes were stimulated with viral antigen (10 µg/10 µl culture medium) for 5 days in a humidified incubator with 5% CO₂ at 37 °C in the presence of 0.25×10^5 autologous antigen presenting cells (APC). Cell proliferation was measured by the addition of 50 µl per well of culture medium containing 1 µCi of methyl-³H-thymidine (New England Nuclear, specific activity 52 Ci/mmol) for the last 16–18 h of culture. Cells were harvested onto fiberglass filter paper with a cell harvester, and incorporated radioactivity was measured in a Beckman LS6000 SE-counter (Beckman, Fullerton, CA). Results are expressed as mean counts per minute (cpm) ± S.D. of triplicate cultures. Control experiments to identify possible mitogenic activity of ALL and PNA were performed using 0.1, 1 and 10 µg/10 µl of culture medium of each lectin. Mumps virus was also used as control antigen (10 µg/10 µl culture medium), under similar conditions as RvP, to confirm the specificity of the lymphoproliferation assays.

2.8. PNA and ALL receptor expression after recall viral antigen

PBMC from infected and naive pigs obtained through Ficoll–Hypaque gradient centrifugation were washed with PBS-A and cultured at 2.5×10^5 per well in sterile 96-wells round bottom plates in 200 µl RPMI culture medium as indicated under lymphoproliferative assays. The cells were stimulated with viral antigen (10 µg/10 µl culture medium) in a humidified incubator with 5% CO₂ at 37 °C. Mumps virus was used as control antigen (10 µg/10 µl culture medium). Cells without stimulation or cultured in presence of RvP, after 48–120 h of stimulation were washed with sterile PBS to eliminate the RvP and suspended in PBS; then, the cells (10^6 per tube) were reacted with biotin–ALL or FITC–PNA. After 20 min incubation on ice, ALL cells were washed with PBS and reacted with PE–streptavidin. After a final wash, cells were suspended in PBS and analyzed in the FACS Excalibur. Results are expressed as the mean proportion of cells positively recognized by the lectin.

3. Results

3.1. PNA⁺ and ALL⁺ lymphocyte subset characterization

To determine the expression of O-glycosylated structures triple-marker immunostaining was performed to analyze the recognition of PNA or ALL lectins. Cellular populations were differentially recognized by ALL and PNA lectins, our results indicated that ALL recognized preferentially CD4⁺CD8⁺ lymphocytes ($P < 0.05$), and PNA recognized both CD4⁻CD8⁻ and CD4⁺CD8⁺ lymphocytes ($P < 0.05$) (Table 1). After 8 weeks of infection, the proportion of PNA⁺ CD4⁺CD8⁺ lymphocytes showed a two-fold increase as compared to non-immunized pigs, and the amount of CD4⁻CD8⁻ was significantly diminished (Table 2). The proportion of ALL⁺CD4⁺CD8⁺ lymphocytes from infected animals diminished with respect to naive pigs ($P < 0.05$), with a slight increase of CD4⁺CD8⁻ ($P < 0.05$; Table 2).

Table 1
Analysis of naive porcine PNA and ALL positive T lymphocytes^a

Subset	Recognized cells (%)	
	PNA	ALL
CD4 ⁺ CD8 ⁻	11 ± 7	11 ± 4
CD4 ⁺ CD8 ⁺	44 ± 6*	42 ± 6*
CD4 ⁻ CD8 ⁺	14 ± 5	27 ± 8
CD4 ⁻ CD8 ⁻	32 ± 4*	27 ± 5

^a Results represent the mean ± S.D. of four independent assays ($n = 4$).

* $P < 0.05$.

Table 2
Analysis of immunized porcine PNA and ALL positive T lymphocytes after viral challenge^a

Subset	Recognized cells (%)	
	PNA	ALL
CD4 ⁺ CD8 ⁻	9 ± 2	25 ± 4*
CD4 ⁺ CD8 ⁺	84 ± 6*	34 ± 8*
CD4 ⁻ CD8 ⁺	14 ± 4	16 ± 7
CD4 ⁻ CD8 ⁻	9 ± 2	25 ± 7*

^a Eight weeks after immunization with 5 ml of 10^4 TCID₅₀ ml⁻¹ of RvP ($n = 7$).

* $P < 0.05$.

3.2. PNA⁺ memory-lymphocytes increased after infection

As indicated in Fig. 1, the analysis of the cells recognized by these lectins indicated that PNA⁺ lymphocytes showed higher rate of the CD29 antigen (PNA⁺CD29^{high}), than the ALL⁺ (ALL⁺CD29^{low}). The number of PNA⁺CD29^{high} lymphocytes increased significantly ($P < 0.01$) after experimental infection with the RvP, and most ALL⁺CD29^{low} cells became CD29^{middle}.

3.3. PNA⁺ cells are responsive to recall viral antigen

To identify the possible participation of PNA⁺ lymphocytes in memory/effector activity, we analyzed the capacity of electronically sorted PNA⁺ and ALL⁺ peripheral blood lymphocytes from RVP-infected pig to mediate a secondary response to viral antigen. Our results showed that both PNA⁺ and ALL⁻ cell populations from RvP-infected pigs incorporated methyl-³H-thymidine nine and five times higher than unstimulated control cells, whereas PNA⁻ and ALL⁺ lymphocytes responded very poorly at stimulation with equal doses (10 µg) of recall viral antigen. Recall antigen stimulation was significantly higher ($P < 0.01$) in PNA⁺ and ALL⁻ than in PNA⁻ and ALL⁺ (Fig. 2). PNA⁺ and ALL⁻ cells from naive pigs were unable to respond to the virus. Stimulation of sorted cells, from infected and uninfected pigs, with mumps virus did not elicit a response (data not shown). Control experiments, using PNA or ALL as mitogens, indicated that these lectins were unable to stimulate proliferation in porcine lymphocytes.

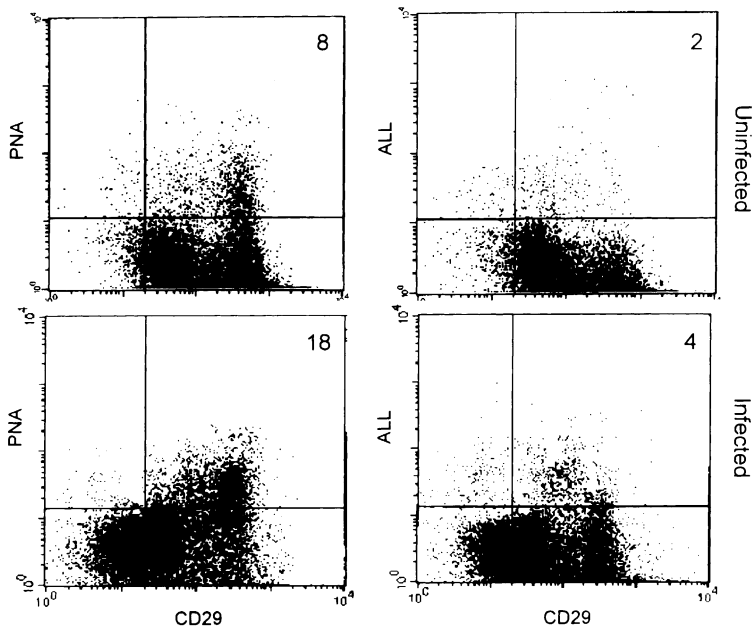


Fig. 1. Two-color cytofluorometric analysis of PBMC. Representative experiment of PBMC from a naive pig (upper) and after 2 months of infection (bottom) stained with biotin-conjugate PNA or ALL lectins and mAb anti-CD29. Numbers represent the percentages of positive cells within each quadrant.

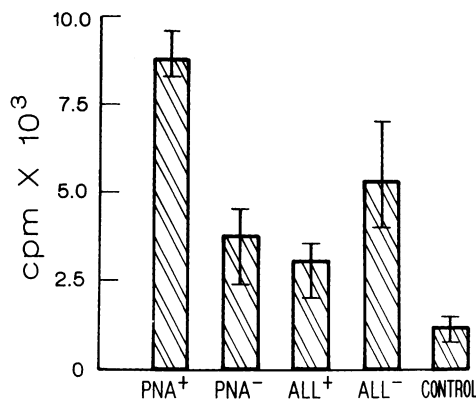


Fig. 2. PNA⁺ lymphocytes respond to a recall viral antigen. PNA⁺, PNA⁻, ALL⁺ and ALL⁻ lymphocytes from infected pigs were electronically sorted and cultured with autologous antigen presenting cells in presence of viral antigen. Proliferative response of flow cytometry-separated T subpopulations was quantified by methyl-³H-thymidine incorporation (cpm). Results are expressed as the mean of three replicates \pm S.E. of the mean, $n = 7$.

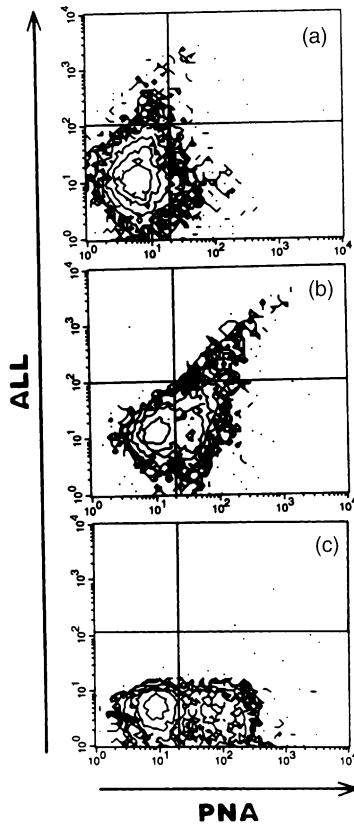


Fig. 3. PNA and ALL receptor expression on porcine lymphocytes after recall viral antigen *in vitro*. PBMC from infected pigs were cultured with RvP and expression of ALL and PNA receptors on cells at 0 h (a), 48 h (b) and 120 h (c) of culture was determined by cytofluorometric assays. The cells (10^6 per tube) were reacted with biotin-ALL or FITC-PNA. ALL⁺ cells were reacted with PE-streptavidin. The cells were analyzed in the FACS Excalibur. Figure is a representative experiment of four.

3.4. PNA⁺ and ALL⁺ cells after recall viral antigen *in vitro*

Expression of PNA and ALL receptors on immunized porcine lymphocytes after RvP stimulation was determined *in vitro*. As indicated in Fig. 3, after RvP stimulation the proportion of ALL⁺ cells diminished from 7.5 ± 2 to $0.5 \pm 0.3\%$ after 48 h stimulation; whereas, the PNA⁺ cells increased from 4 ± 1 to $42 \pm 2\%$. After 48 h of antigen recall, we observed a PNA⁺ALL⁺ cell population corresponding to $4 \pm 0.5\%$ (Fig. 3); however, after 72–120 h, ALL⁺ and PNA⁺ALL⁺ cells disappear, whereas PNA⁺ cells remained constant ($34 \pm 5\%$). Control experiments performed on lymphocytes from infected pigs incubated in presence of mumps virus, showed no effect on expression of ALL or PNA receptors; moreover, the proportion on the expression of PNA or ALL receptors on lymphocytes from non-infected pigs was not modified after RvP stimulation (not shown).

3.5. Sugar specificity of PNA⁺ and ALL⁺

In all analyses, we performed inhibition assays as control experiments, using 200 mM Gal for PNA and GalNAc for ALL. Lectin interactions were completely abolished in all assays.

4. Discussion

The glycosylation pattern on T cell surface glycoproteins changes when cells in the thymus mature and migrate to the periphery (Gillespie et al., 1993). In swine, as in other animal species, maturation of T cells is assessed by expression of the T cell receptor and the CD4⁺ and CD8⁺ co-receptor molecules; furthermore, swine CD4⁺CD8⁺ lymphocytes exhibit properties of mature antigen experienced-cells (Summerfield et al., 1996; Zuckermann and Husmann, 1996; Hernández et al., 2001). For this reason it would be useful to identify unique oligosaccharides that characterize T cell subsets, especially if these structures can be used to distinguish memory and effector T cells from naive T cells. In this study, we showed that porcine lymphocytes expressing PNA⁺ became CD29^{high}, whereas naive CD29^{low} lymphocytes were recognized by the lectin from *A. leucocarpus* seeds, which might indicate either altered expression or changes in the three-dimensional organization of *O*-glycans during the activation of porcine T cells.

RvP-induced activation produced an expansion of PNA⁺ CD4⁺CD8⁺ cells. The increase in PNA binding correlated with an increase of cells that were also CD29^{high}. The proportion of ALL⁺ CD29^{low}, which seems to correspond to naive cell subsets, remained unaltered after RvP infection. Porcine CD29 is the β 1 subunit of the integrin that functions as a receptor for cell adhesion molecules of the extracellular matrix; it is involved in rejection of pig-to-human tissue xenografts and in homing and differentiation of hematopoietic progenitor cells (Jimenez-Marin et al., 2000). These data suggested that increase in PNA binding of T cells is a property of memory T cell activation during viral infection and also suggested that porcine CD4⁺CD8⁺ lymphocytes have memory functions (Summerfield et al., 1996; Zuckermann and Husmann, 1996; Hernández et al., 2001).

The memory phenotype on PNA⁺ lymphocytes suggested that PNA⁺ cells might include memory/effector T cells, and the naive phenotype on ALL⁺ lymphocytes might contain cells unable to respond in a viral-antigen assay. To prove this hypothesis, the capacity of electronically sorted PNA⁺ and ALL⁺ peripheral blood lymphocytes to mediate a secondary response to RvP was analyzed. Cytofluorometric analyses of sorted PNA⁺, PNA⁻, ALL⁺, and ALL⁻ lymphocytes showed that both PNA⁺ and ALL⁻ cell populations from RVP-infected pigs were able to respond to stimulation with equal doses of recall viral antigen, whereas PNA⁻ and ALL⁺ lymphocytes from RVP-infected pigs were unable to respond. As demonstrated in murine CD8⁺ T cells (Harrington et al., 2000), our results confirm that porcine PNA⁺ and ALL⁻ cells contain memory/effector T cells populations and that ALL⁺ cells include quiescent/naive cells.

The specificity of ALL is directed to GalNAc in the inner core of type 1 *O*-glycosidically linked glycans, such as T (Gal β 1, 3GalNAc-Ser/Thr) and Tn antigens (GalNAc α 1,0 Ser/Thr) (Zenteno et al., 1992); although, PNA shows similar specificity for the OH⁻ from C4 of

Gal in the same T antigen, it has been demonstrated that this lectin can also interact with Gal residues present in the Gal β 1, 4GlcNAc substitutions identified on type 2 *O*-glycosidically linked glycans on CD43 and CD45 from activated T cells (De Maio et al., 1986; Pereira et al., 1976; Wu et al., 1996); as well as in type 2 *O*-glycans on the CD8 chain from activated T cells (Casabo et al., 1994; Galvan et al., 1998). The level of interaction of PNA with CD29^{high} T cells increased significantly in infected animals. It is possible that PNA staining might identify cell surface molecules able to distinguish memory from naive cells (Harrington et al., 2000). Our findings show the presence of a cellular subset that corresponds to naive or probably quiescent T cell populations, since they showed to be ALL⁺ and CD29^{low}. The murine lymphocyte receptor for ALL is a glycoprotein of 70 kDa, it is a typical sialylated *O*-glycosylprotein, and has low (<17%) homology with the Fas-associated death domain protein, and transforming growth factor- β type II receptor (Porrás et al., 2000). The lymphocyte *O*-linked glycans reactive with ALL are present in murine medullar lymphocytes (Lascurain et al., 1994) and in human T cells with the CD4⁺CD45RA⁺CD27⁺ phenotype, indicating that the lectin receptor is present specifically in naive or quiescent cell subpopulations (Lascurain et al., 1997).

The *O*-glycosylation changes that occur in porcine lymphocytes documented in this study could be important for trafficking and localization of memory/effector cells to sites of antigen presentation. Alterations in cell surface glycosylation may also affect T cell recognition of APCs, or interactions with accessory molecules (Kijimoto-Ochiai and Uede, 1995). The dynamic modulation of T cell surface glycosylation indicates that these changes affect T cell function, in addition to allowing phenotypic discrimination between naive and effector/memory T cells (Harrington et al., 2000; Lascurain et al., 1997; Porrás et al., 2000).

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