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Phenotypic classification of porcine lymphocyte subpopulations in blood and lymphoid tissues

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SUMMARY

The pig is a useful model for the heterogeneity of the mammalian immune system and has also recently received attention as a possible source of organs for human transplantation. Here we report a detailed analysis of porcine lymphocyte phenotypes. Peripheral blood $\alpha\beta$ T cells consisted of four subsets ($CD4^+8^-$, $CD4^+8^{lo}$, $CD4^-8^{lo}$ and $CD4^-8^{hi}$) and $\gamma\delta$ T cells of three ($CD2^-4^-8^-$, $CD2^+4^-8^{lo}$ and $CD2^+4^-8^-$). There were, in addition, a large proportion of non-T-non-B lymphocytes with $CD2^+3^-4^-8^{lo}$ surface immunoglobulin-negative phenotype containing natural killer (NK) activity. A striking observation was the relatively low frequency of $\alpha\beta$ T cells in the blood of young pigs. Similar phenotypes were also identified in the cells from peripheral lymphoid tissues, though the proportions of the $\gamma\delta$ T cells and the non-T-non-B lymphocytes in the lymph nodes and tonsil were much lower and the majority of the $\gamma\delta$ T cells in the lymphoid tissues bore CD2 and/or CD8. In thymus, the small thymocytes were predominantly $CD3^-4^+8^+$while the mature large thymocytes displayed phenotypes similar to those of peripheral T cells. Thus this work has directly defined porcine $\alpha\beta$ and $\gamma\delta$ T cells, demonstrated the T-cell nature of the unique $CD4^+8^+$ subset of peripheral lymphocytes, revealed the high heterogeneity of the $CD8^+$ cells, and established the phenotype of NK cells. The functional properties of these defined porcine lymphocyte subsets can now be experimentally determined in health and disease.

INTRODUCTION

The usefulness of the pig as an experimental animal is increasingly appreciated with the realization of its potential applications in xeno-transplantation.[1] Furthermore, the pig also possesses many fascinating immunobiological features, such as the unique inverted lymph node structure, an unusual route of lymphocyte recirculation[2] and the unusual range of cell populations in the peripheral blood T-lymphocyte pool. Like chickens and ruminants, porcine peripheral blood contains a large proportion of $\gamma\delta$ T-cell receptor (TCR)-bearing cells ($\gamma\delta$ T cells). Furthermore, porcine peripheral lymphocytes have a high proportion of a $CD4^+CD8^+$ T-cell subset which is not detected in significant numbers in other species.[3–5] The existence of these unconventional double-positive cells in the periphery poses a challenge to the current understanding of the functional roles of the CD4 and CD8 molecules in antigen recognition by helper and cytotoxic T cells, respectively. Therefore, the porcine immune system provides unique opportunities for exploring the nature and physiological functions of specialized lymphocyte subpopulations, which are rare or inaccessible in man and mouse.

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Due to the lack of appropriate surface markers, however, classification of the porcine lymphocytes has been difficult. In the absence of specific anti-CD3 and TCR monoclonal antibodies (mAb), porcine T cells have been identified through the expression of CD2, CD4, CD8, the absence of surface immunoglobulin (sIg) and the non-adherence to nylon wool (reviewed in ref. 5). Such indirect approaches have a number of limitations. For example, CD2 was also found to be expressed on non-T cells, such as NK cells,[6] and some B cells (our unpublished data). The currently available markers for the porcine $\gamma\delta$ T cells (CD4 plus CD8,[7,8] 86D,[9] MAC320,[10] CC101[11]) mainly identify $CD2^-$ surface immunoglobulin-negative or $CD4^-8^-$ surface immunoglobulin-negative cells, yet we know in other species, some $\gamma\delta$ T cells do express CD2 and/or CD8. The confused phenotypic classification of porcine T-cell subpopulations is therefore in sharp contrast to the progress in the molecular biological studies as the cDNA encoding $\alpha\beta$ and $\gamma\delta$ TCR have both been cloned in this important species.[12]

In order to solve this problem, we have prepared two key reagents. One is directed against the porcine CD3 ε-chain[13] (mAb PPT3) and thus identifies total T cells. The other (mAb PPT19) is directed to a novel CD3 epitope which is associated only with the 43 000 MW $\gamma\delta$ dimer, but not the 55 000 MW $\alpha\beta$ dimer, of TCR (ref. 13 and H. Yang and R.M.E. Parkhouse, manuscript in preparation) and hence can be used a specific $\gamma\delta$

T-cell marker. Using these two reagents, we have been able to resolve the complexity of porcine lymphocytes into four $\alpha\beta$ T-cell subsets, three $\gamma\delta$ T-cell subsets and three non-T-non-B-cell subsets. We have also analysed the phenotypes of lymphocyte subpopulations from lymphoid organs including the thymus. This phenotypic information provides a clue in elucidating both the ontogeny and functions of these cells. As a result, one of the peripheral blood lymphocyte subsets has been found to contain natural killer (NK) activity.

MATERIALS AND METHODS

Monoclonal antibodies and reagents

Monclonal antibodies to the porcine CD3 ε-chain (PPT3, mouse IgG1) and the $\gamma\delta$ T cell-specific CD3-related antigen (PPT19, mouse IgG1) are to be described elsewhere, respectively (ref. 13 and H. Yang and R.M. E. Parkhouse, in preparation). The following anti-porcine lymphocyte mAb have previously been described: anti-CD2, MSA4 (IgG2a);[14] anti-CD4, 74-12-4 (IgG2b, κ);[15] anti-CD8; 11/295/33 (IgG2a, κ);[16] anti-monocyte-granulocytes, 74-22-15 (IgG1, κ).[15] To identify B cells, fluoroscein isothiocyanate (FITC)-conjugated goat anti-porcine immunoglobulin (Southern Biotechnology Association, Inc, Birmingham, AL) was used after being absorbed with immobilized murine immunoglobulin to remove cross-reacting antibodies. FITC- or phycoerythrin (PE)-labelled goat anti-murine subclass immunoglobulin antibodies and streptavidin–PE/CY.5 were purchased from Southern Biotechnology Association, Inc.

Lymphocyte preparation

Young outbred pigs (aged 4 weeks) were used as donors of both blood and lymphoid tissues whilst older pigs (4–16 months) were only donors of blood. The 4-week-old pigs were bled under anaesthesia (4 ml Sagatal via cardiac), while pigs of the other age group were bled without anaesthesia. Preparation of lymphocytes has been described.[17] Briefly, peripheral blood mononuclear cells (PBMC) from citrated blood were prepared by dextran sedimentation of red cells followed by step gradient centrifugation using Lymphoprep (Nycomed Pharma As, Oslo, Norway). Single lymphoid cell suspensions were prepared by teasing lymphoid tissues into ice-cold phosphate-buffered saline (PBS) containing 2% (v/v) fetal calf serum (FCS) before gradient separation. The contaminating red cells were lysed by treatment with 0·15 M, pH 7·4 NH_4Cl solution for 5 min at room temperature. Purified PBMC were suspended in PBS containing 2% (v/v) FCS and 0·1% (w/v) NaN_3 for flow cytometric analysis (FCM).

For NK cytotoxic assay, citrated porcine blood was incubated with carbonyl iron at 37° for 45 min to remove monocytes magnetically[6] before further separation as describe above. Cell subsets were selectively depleted from purified peripheral blood lymphocytes (PBL) by panning as described previously.[18] Briefly, plastic Petri dishes (10 cm, Bibley Sterilin Ltd, Stone, Staffs, UK) were coated with 15 ml of goat anti-mouse immunoglobulin solution (10 μg/ml in PBS, Sigma) at 4° overnight. The dishes were loaded with PBL [2×10^7 cells in PBS–2% (v/v) FCS (10 ml)] which had been coated with the mAb as indicated in the text. After 1 hr of incubation at 4°, unbound cells were recovered and used as effector cells in the NK assay.

Three-colour FCM

The three-colour FCM analysis was carried out using FITC- and PE-conjugated anti-murine immunoglobulin subclass antibodies as the first and second colour reagents respectively, with streptavidin–PE/CY.5 as the third colour. For this, 10^6 fresh PBMC were mixed with pairs of mAb of different subclasses, followed by staining with mixtures of FITC- and PE-conjugated anti-subclass antibodies. The cells were then treated with 10% (v/v) normal mouse serum to block unoccupied binding sites on the second antibodies, and then stained with biotinylated mAb and streptavidin–PE/CY.5. All incubations were done for 30 min at 4°. Cold PBS/FCS/NaN_3 mentioned above was used for all of the washing and staining operations. The data were acquired for 25 000 cells and analysed using the LYSIS II program on a FACScan cytometer (Becton Dickinson, USA). Monocytes were gated out according to their forward scatter and side scatter parameters which were defined by anti-monocyte-granulocyte mAb 74-22-15-reactive cells. Residual contaminating monocytes were finally excluded in data analyses based on mAb 74-22-15 staining (see text).

In the phenotyping analysis, total T cells were identified with anti-CD3 mAb PPT 3, B cells with FITC-conjugated anti-porcine immunoglobulin, $\gamma\delta$ T cells with anti-$\gamma\delta$ T-specific CD3 mAb PPT 19, and $\alpha\beta$ T cells by the phenotype of pan-$CD3^+$ $\gamma\delta$ T-specific $CD3^-$.

NK cytotoxic assay

This has been described previously.[6] Briefly, K562 cells growing in log phase were labelled with ^{51}Cr and used as target cells. Target cells at 10^5/ml in RPMI-1640 containing 10% (v/v) FCS, penicillin and streptomycin at 100 unit/ml and 100 μg/ml, respectively were mixed with an equal volume of effector cells. Aliquots of 200 μl of the mixture were placed in 96-well plates (flat bottom) and cultured under standard conditions for 18 hr. Each treatment was in triplicate. After incubation, 100 μl of supernatant was removed and counted for radioactivity using a gamma-counter. Background release was that of target cells mixed with an equal volume of medium instead of effector cells. Maximal release was determined by mixing 100 μl targets with 100 μl 5% (v/v) Triton X-100. The specific release was determined by the following formula:

$$\% \text{ specific-release} = \frac{\text{c.p.m.}_{\text{experiment}} - \text{c.p.m.}_{\text{background}}}{\text{c.p.m.}_{\text{maximum}} - \text{c.p.m.}_{\text{background}}}$$

RESULTS

Phenotypes of PBL subsets

The phenotype of $\alpha\beta$ T cells is shown in Fig. 1(a) and 1(b). When $\gamma\delta$ T-specific CD3 expression was plotted versus pan-CD3 expression, PBL were resolved into three regions (Fig 1a, left). The cells in the upper-right quadrant were $\gamma\delta$ T cells which expressed both pan-CD3 marker and $\gamma\delta$ T-specific CD3. The cells in the lower-right quadrant, by contrast, expressed only pan-CD3 marker but not $\gamma\delta$ T-specific CD3 and therefore by inference were $\alpha\beta$ T cells. When $CD2^-$ cells were gated in the analyser, most of them were found to be either $\gamma\delta$ T cells or non-T cells, but not $\alpha\beta$ T cells (Fig. 1a, middle). In other words,

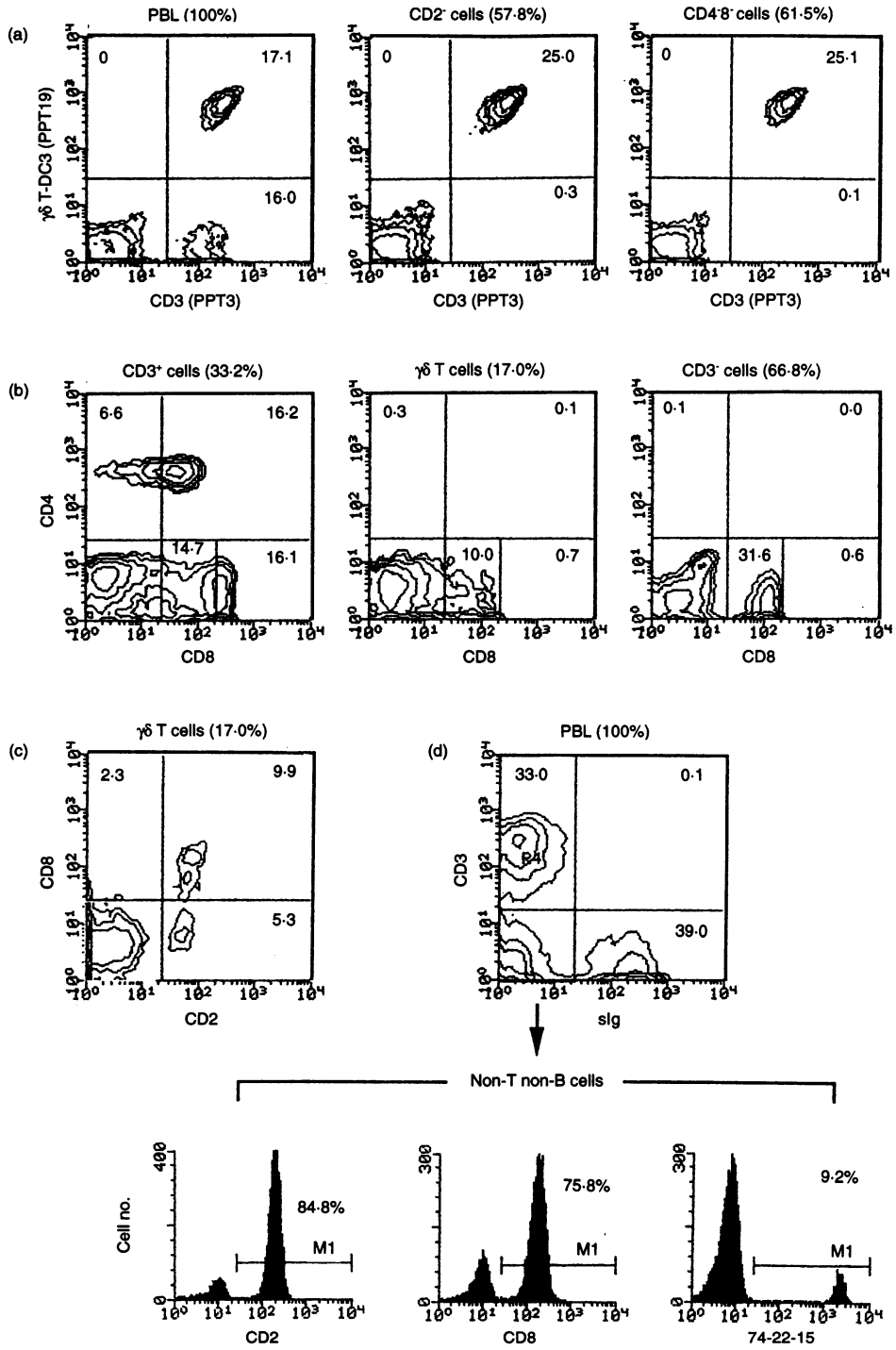

Fig. 1. Triple staining FCM profiles of PBL of 4-week old pigs. (a) PBL were coated with a mixture of anti-$\gamma\delta$ T-CD3 (mAb PPT19, IgG1) and either anti-CD2 (IgG2a) or anti-CD4 (IgG2b) plus anti-CD8 (IgG2a) followed by a mixture of PE–anti-mouse IgG1, FITC–anti-mouse IgG2a and FITC–anti-mouse IgG2b. After treatment with 10% normal mouse serum (NMS), the cells were further stained with biotinylated anti-pan CD3 (mAb PPT3, IgG1) followed by streptavidin PE/CY5. $CD2^-$ or $CD4^-8^-$ cells were gated in the analyser as indicated. (b) PBL were stained with anti-CD4 (IgG2b) plus anti-CD8 (IgG2a) followed by PE–anti-mouse IgG2b plus FITC–anti-mouse IgG2a, treated with 10% NMS, and further stained with either biotinylated anti-pan CD3 or biotinylated anti-$\gamma\delta$ T-CD3 followed by streptavidin-PE/CY5. Pan-$CD3^+$, $CD3^-$ and $\gamma\delta$ T-$CD3^+$ cells were gated in the analyzer as indicated. (c) PBL were stained with anti-CD8 (IgG2a) plus anti-$\gamma\delta$ T CD3 (IgG1) followed by PE–anti-mouse IgG1 plus FITC–anti-mouse IgG2a, treated with 10% NMS and stained with biotinylated anti-CD2 followed by streptavidin PE/CY5. $\gamma\delta$ T-$CD3^+$ cells were gated for analysis. (d) PBL were stained with FITC–anti-pig-immunoglobulin plus either anti-CD2 (IgG2a), anti-CD8 (IgG2a) or granulocyte-monocyte specific mAb 74-22-15 (IgG1) followed by PE–anti-mouse IgG1 plus PE–anti-mouse IgG2a, treated with 10% NMS and stained with biotinylated anti-CD3 followed by streptavidin PE/CY5. $CD3^-$ surface immunoglobulin-negative cells (lower left quadrant) cells were gated for analyses of the expression of the third marker as indicated.

Table 1. Composition of lymphoid cells from young (4-week-old) pigs; porcine lymphocytes were stained and cell subsets were identified and enumerated using flow cytometric analysis as described in Fig. 1. Values are mean percentage of total cells ± SD ($n = 7$)

	Tissue				
Subset	PBL	Spleen	PLN	MLN	Tonsil
$\alpha\beta$-T(2^+)	12·0 ± 3·7	22·9 ± 7·9	54·0 ± 6·7	55·0 ± 3·6	23·1 ± 5·2
4^+8^-	2·1 ± 0·9	8·9 ± 4·3	33·3 ± 4·8	26·3 ± 3·6	10·5 ± 2·9
4^+8^{lo}	2·2 ± 1·4	7·3 ± 2·9	8·7 ± 2·9	9·9 ± 2·3	7·6 ± 2·3
4^-8^{lo}	3·7 ± 1·4	3·0 ± 1·3	6·8 ± 2·0	6·7 ± 2·6	3·1 ± 2·0
4^-8^{hi}	4·0 ± 1·5	3·8 ± 2·1	5·0 ± 1·6	12·1 ± 2·8	1·9 ± 0·8
$\gamma\delta$-T(4^-)	23·9 ± 6·5	26·2 ± 6·2	6·3 ± 1·4	3·7 ± 1·3	2·5 ± 0·7
2^+8^{lo}	2·2 ± 1·0	9·0 ± 4·0	2·7 ± 2·1	2·4 ± 1·2	1·0 ± 0·3
2^+8^-	1·0 ± 1·0	13·1 ± 9·0	0·2 ± 0·1	0·2 ± 0·1	0·2 ± 0·2
2^-8^-	20·7 ± 5·0	4·2 ± 1·9	3·4 ± 1·2	1·1 ± 0·4	1·3 ± 0·5
Non-T-B(4^-)	31·7 ± 12·0	16·3 ± 2·5	8·2 ± 3·3	6·3 ± 2·6	17·5 ± 3·9
2^+8^{lo}	27·7 ± 13·3	12·3 ± 4·1	3·7 ± 2·0	2·3 ± 2·6	2·0 ± 2·2
2^+8^-	2·3 ± 1·7	2·3 ± 0·6	2·4 ± 0·9	1·8 ± 0·7	5·0 ± 4·3
2^-8^-	1·8 ± 1·0	1·7 ± 1·3	2·1 ± 1·9	2·2 ± 1·0	10·5 ± 4·6
B	32·4 ± 12·8	34·6 ± 6·4	31·5 ± 4·4	35·0 ± 2·8	56·9 ± 7·5

most of the $\alpha\beta$ T cells expressed CD2. Similarly, most $\alpha\beta$ T cells were found to express CD4 and/or CD8 (Fig 1a, right).

When CD4 expression was plotted versus CD8, total $CD3^+$ T cells, when gated in the analyser, were resolved into five subsets. They were $CD4^+8^-$, $CD4^+8^{lo}$, $CD4^-8^-$, $CD4^-8^{lo}$, and $CD4^-8^{hi}$ (Fig. 1b, left). In contrast, the $\gamma\delta$ T cells were either $CD4^-8^-$ or $CD4^-8^{lo}$ (Fig. 1b, middle). Since $CD4^+8^-$, $CD4^+8^{lo}$ and $CD4^-8^{hi}$ cells expressed pan-CD3 but not the $\gamma\delta$ T-cell-specific CD3 antigen (Fig. 1b, left and middle), and non-T cells were absent in these regions (Fig. 1b, right), all the $CD4^+$ and $CD8^{hi}$ cells in PBL must be $\alpha\beta$ T cells. Similar proportions of $CD4^-8^-$ cells were found to express pan-$CD3^+$ or $\gamma\delta$ T-specific $CD3^+$ (14·5% of PBL, Fig. 1b, left and middle). In addition, as shown in Fig. 1(a), effectively no $\alpha\beta$ T cells were $CD4^-8^-$. Therefore, $CD3^+4^-8^-$ cells contained only $\gamma\delta$ T cells. By contrast, $CD3^+4^-8^{lo}$ cells included both $\alpha\beta$ and $\gamma\delta$ T cells. The proportion of $\alpha\beta$ T cells with this phenotype was calculated by subtraction of the proportion of the $\gamma\delta$ T cells (1·7% of PBL) from that of total CD3 cells in this region (4·8% of PBL). Taken together, the phenotype of $\alpha\beta$ T cells include $CD4^+8^-$, $CD4^+8^{lo}$, $CD4^-8^{lo}$, and $CD4^-8^{hi}$, all of them being $CD2^+$.

Having found that all the $\gamma\delta$ T cells were $CD4^-$, we further investigated the CD2 and CD8 expression of the gated $\gamma\delta$ T cells. As shown in Fig. 1(c), $\gamma\delta$ T cells were resolved into three phenotype groups: most of them were $CD2^-8^-$, but $CD2^+8^{lo}$ and $CD2^+8^-$ subpopulations were also present.

The phenotype of the non-T-non-B cells was analysed in Fig. 1(d). When CD3 expression in PBL was plotted versus surface immunoglobulin expression, non-T-non-B cells were defined in the lower-left quadrant (Fig. 1d). The majority of these cells express CD2 (23·6% of PBL) and CD8 (21·1%). A small proportion of the cells in this region (2·6%) were stained by the monocyte-granulocyte-specific mAb 74-22-15. Since most of the $CD8^+$ cells were also $CD2^+$, it seems reasonable to resolve cells in this region into three subsets, namely, $CD2^+8^{lo}$ (21·1% of the total), $CD2^+8^-$ (2·5% of the total) and $CD2^-8^-$ (4·3% of the total). As the mAb 74-22-15 does not stain $CD2^+$ or $CD8^+$ cells (data not shown), the contaminating monocyte should be present in $CD2^-8^-$ cells, and its proportion was discounted from calculation and the percentage of cell subsets presented in Table 1 was adjusted accordingly. The size (in terms of forward scatter) and granular contents (side scatter) of the non-T-non-B lymphocytes were found in the same range as those of the T or B cells (data not shown).

Taken together, the employment of staining protocol and FCM analysis presented above resolved porcine PBL into 11 subsets with different phenotypes. Table 1 summarizes the frequency of these 11 phenotypes in the peripheral blood of young (4-weeks-old) pigs; average of seven separate experiments.

A remarkable feature of the peripheral T-cell composition of these young pigs is the high proportion of $\gamma\delta$ T versus $\alpha\beta$ T cells ($P < 0{\cdot}01$). To investigate whether this was a unique feature of young pigs, similar analyses were carried out using PBL from pigs of different age groups. Inspection of the data reveals a clear trend of a higher proportion of $\alpha\beta$ T cells in old versus young pigs. For example, compared to the 4-week group, the proportion of $\alpha\beta$ T cells was significantly higher in the 8-month ($P < 0{\cdot}01$), 12-month ($P < 0{\cdot}001$) and 16-month ($P < 0{\cdot}01$), but not in the 4-month groups (Tables 1 and 2). Restricting the comparison to the 4-month group still reveals the same trend: both the 12- and 16-month-old pigs had higher proportions of $\alpha\beta$ T cells than the 4-month-old pigs ($P < 0{\cdot}01$ and $P < 0{\cdot}05$, respectively). On the other hand, the percentage of the $\alpha\beta$ T cells in the 16-month group was not significantly different from the 12-month group, suggesting that the increase in the $\alpha\beta$ T-cell proportion reaches a plateau at this stage (Table 2). A consistent finding was the decreasing proportion of non-T-non-B and B cells with age, whereas the percentage of $\gamma\delta$ T cells remained more or less constant (Tables 1 and 2).

Table 2. Composition of PBL from pigs of different age groups; values are mean percentage of total cells ± SD. The numbers of the animals tested are indicated

	Age			
Subset	4 months ($n=3$)	8 months ($n=4$)	12 months ($n=3$)	16 months ($n=4$)
$\alpha\beta$-T(2^+)	14·2 ± 2·8	30·0 ± 11·3	48·8 ± 11·3	34·2 ± 12·5
4^+8^-	3·4 ± 2·5	7·0 ± 2·9	14·3 ± 5·7	4·3 ± 2·7
4^+8^{lo}	3·7 ± 0·5	4·9 ± 1·1	6·8 ± 2·7	9·6 ± 4·8
4^-8^{lo}	3·4 ± 1·0	7·8 ± 4·0	15·5 ± 2·5	9·9 ± 2·9
4^-8^{hi}	3·7 ± 0·7	10·3 ± 5·9	12·2 ± 4·5	10·4 ± 3·4
$\gamma\delta$-T(4^-)	39·6 ± 7·2	36·8 ± 13·8	21·4 ± 1·9	31·6 ± 14·1
2^+8^{lo}	6·6 ± 3·4	2·9 ± 0·5	4·4 ± 1·7	8·4 ± 3·1
2^+8^-	1·5 ± 0·7	1·2 ± 0·6	1·9 ± 0·7	0·4 ± 0·1
2^-8^-	31·5 ± 7·7	32·7 ± 13·6	15·1 ± 1·7	22·8 ± 13·9
Non-T-B(4^-)	14·9 ± 11·0	8·0 ± 4·6	6·9 ± 2·7	10·9 ± 4·4
2^+8^{lo}	9·9 ± 5·9	7·0 ± 4·7	5·0 ± 3·2	8·3 ± 5·4
2^+8^-	3·2 ± 3·0	0·4 ± 0·7	0·4 ± 0·5	0·4 ± 0·5
2^-8^-	1·8 ± 2·4	0·6 ± 0·6	1·5 ± 0·9	2·2 ± 1·3
B	31·3 ± 6·9	25·3 ± 8·8	22·9 ± 11·8	23·4 ± 5·9

It should be noted that with the exception of the 4-week-old animals (Table 1), which were anaesthetized before bleeding, all others (Table 2) were bled without anaesthetic. Since a similar low proportion of $\alpha\beta$ T cells was observed both in the 4-week and 4-month groups, it is unlikely that the increase in $\alpha\beta$ T-cell proportion observed in old pigs is an artefact caused by anaesthesia. Also, without determination of the absolute number of lymphocytes, the data presented only reflect the change in T-cell subset percentage with ageing.

We have observed that $CD4^-8^-$ T cells express CD3 at a higher level than the T cells expressing CD4 and/or CD8 cells.[13] In this study, the expression of CD3 in T-cell subsets was also investigated using the statistics data of the FCM analyses presented in Table 1. The mean fluorescence intensity (MFI) of the $CD4^-8^-$ $\gamma\delta$ T cells, being the highest, was arbitrarily defined as 100% and thus served as the standard for quantitative comparison of relative CD3-expression in directly identifiable T-cell subsets. The calculated relative % MFI of these cell subsets was as follows: $CD4^+8^-$ $\alpha\beta$-T cells, 63·4; $CD4^+8^{lo}$ $\alpha\beta$-T cells, 50·3; $CD4^-8^{hi}$ $\alpha\beta$-T cells, 55·6; $CD4^-8^{lo}$ $\gamma\delta$-T cells, 74·9; $CD4^-8^-$ $\gamma\delta$ T cells, 100. It can be seen that the CD3 expression of $CD4^-8^-$ $\gamma\delta$ T cells was higher than the $CD4^-8^{lo}$ $\gamma\delta$ T cells and $CD4^+8^-$ than $CD4^+8^{lo}$ cells. Although the differences were apparently not statistically significant due to high standard deviations, this staining hierarchy was invariably found in each animal.

Phenotypes of peripheral lymphoid tissue subsets

Similar staining and FCM analyses were also carried out using lymphoid cells from spleen, peripheral lymph nodes (PLN, superficial inguinal lymph nodes used here), mesenteric lymph nodes (MLN) and tonsil. The staining profiles are not shown and only statistical data from seven young pigs (4 weeks old) are summarized in Table 1. All the phenotypes described above could also be found in these tissues with some distinct characteristics.

For the spleen, the proportions of cell subpopulations were most similar to that of PBL and characterized by the high proportion of $\gamma\delta$ T cells and non-T-non-B lymphocytes. However, the percentage of $\alpha\beta$ T cells ($P < 0{\cdot}01$), in particular, the $CD4^+$ cells ($P < 0{\cdot}001$), is higher than that in PBL. Also, the composition of $\gamma\delta$ T cells was quite different in that most ($P < 0{\cdot}001$) express CD2 and/or CD8, contrasting with peripheral blood $\gamma\delta$ T cells where the majority ($P < 0{\cdot}001$) were $CD2^-8^-$ (Table 1).

In PLN and MLN, $\alpha\beta$ T cells were the major T cells ($P < 0{\cdot}001$) of which the majority ($P < 0{\cdot}001$) expressed CD4. The $\gamma\delta$ T cells accounted for only a small proportion. The proportion of the non-T-non-B cells was also lower than in the blood ($P < 0{\cdot}001$) (Table 1).

The composition of tonsil cells was quite similar to the lymph node cells. The only difference was that they contained a considerable number of non-T-non-B cells. Furthermore, unlike such cells detected in the blood and spleen, a higher ($P < 0{\cdot}001$) number of these cells did not express CD2 nor CD8 (Table 1).

Phenotypes of thymocytes

Previous studies have demonstrated that porcine thymocytes consist of two overlapping yet discernable subpopulations with different sizes. The smaller thymocytes are mainly immature thymocytes and the larger contain mature thymocytes.[13,17,19] This phenomenon has been repeatedly observed in approximately 80 pigs, no matter which thymic lobe, and using cell suspensions prepared with or without gradient separation (data not shown). Their phenotype analysis is summarized in Fig. 2 and Table 3.

In contrast to peripheral blood T cells, the majority

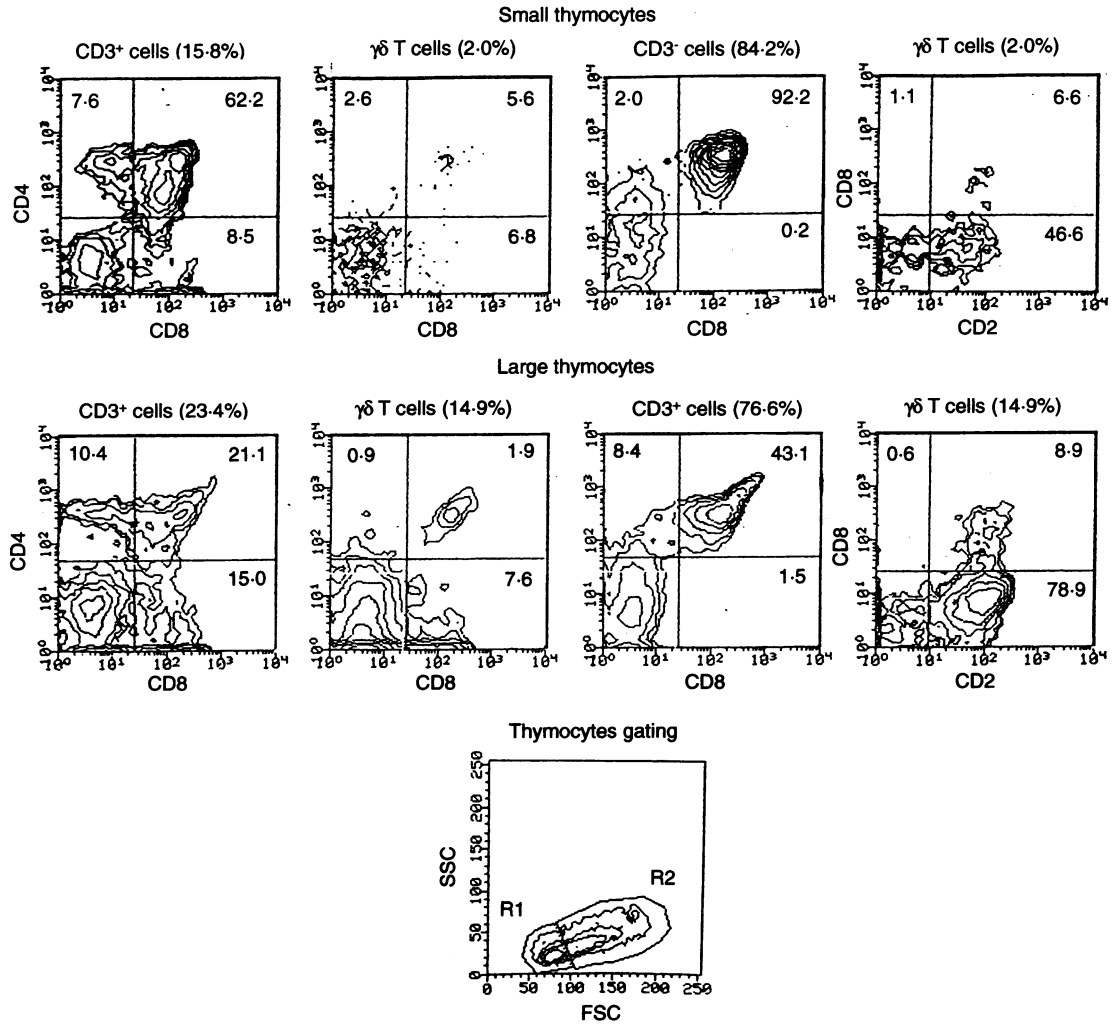

Fig. 2. Triple staining FCM profiles of small and large thymocytes of 4-week-old pigs. Thymocytes were stained with three-colour reagents in similar combinations to those used in Fig. 1(b) and 1(c). Data of the two thymocyte subpopulations were acquired separately on their size (forward scatter, FSC) versus granular contents (side scatter, SSC). Small thymocytes were gated in region 1 (R1) whereas large thymocytes in region 2 (R2) as indicated in the figure.

($P < 0{\cdot}01$) of $CD3^+$ small thymocytes were $\alpha\beta$ T cells which express both CD4 and CD8. Similarly, the $CD3^-$ cells were predominantly $CD4^+8^+$ cells ($P < 0{\cdot}001$) (Fig. 2 and Table 3). Combined with the previous discovery that the $CD3^+$ small thymocytes expressed a low density of CD3 molecule,[13] these features suggest that the small thymocytes are immature and that the majority of them may be programmed to die *in situ*. With this in mind, it is possible that the $CD4^+8^+$ cells found in periphery may not originate from the small thymocytes with similar phenotype.

The composition of the large thymocytes, however, was more similar to that of peripheral blood T cells. Although $CD4^+8^+$ cells still accounted for a large proportion of total $CD3^+$ cells, the major ($p < 0{\cdot}05$) $CD3^+$ subset was $CD4^-8^-$ $\gamma\delta$ T cells. Also, more single-positive $\alpha\beta$ T cells ($CD4^+8^-$ and $CD4^-8^+$) were revealed (Fig. 2). As in the blood, the $\gamma\delta$ T cells were mostly $CD4^-8^-$, plus a small proportion of $CD4^-8^+$ cells. In contrast to the peripheral blood $\gamma\delta$ T cells, however, a large number of $\gamma\delta$ T cells in the thymus expressed CD2. Also, as shown in Fig. 2, a small number of thymic $\gamma\delta$ T cells was often found to be $CD4^+8^+$ cells. Whether these cells are not to be exported to the periphery or would lose CD2 or CD4 before emigration is unclear.

The surface phenotype of peripheral blood NK cells is $CD2^+3^-8^{lo}$

The above analyses reveal the presence of a large proportion of $CD2^+3^-8^{lo}$ cells in the blood. To investigate whether NK cells were present in this cell subset, porcine PBL were depleted of certain subsets by panning and tested for the NK activity. As shown in Table 4, depletion of $CD2^+$ or $CD8^+$ cells from PBL abolished the NK activity. By contrast, removal of $CD4^+$ cells had no significant effects, whereas NK activity was enriched by depletion of the $CD3^+$. The effects were not due to inhibition or activation of the residual positive cells by the selecting mAb since treatment of PBL with anti-CD2, CD3, CD4, or CD8 showed no effects on NK activity (data not shown). Thus, porcine NK cells should express CD2 and CD8, but not CD3 nor CD4. In other words, the NK cells are present in the peripheral blood non-T-non-B cells with a $CD2^+3^-8^{lo}$ phenotype.

Table 3. Composition of thymocytes of young pigs; values are mean percentage of total cells ± SD (n=7).

Subset	Small thymocytes	Large thymocytes
$\alpha\beta$-T(2^+)	22·9 ± 15·3	19·4 ± 8·0
4^+8^-	0·6 ± 0·4	4·8 ± 3·8
4^+8^+	20·1 ± 13·1	11·4 ± 5·5
4^-8^+	2·2 ± 2·1	3·2 ± 1·0
$\gamma\delta$-T	1·4 ± 1·0	18·3 ± 2·9
2^+8^{lo}	0·1 ± 0·1	2·0 ± 0·8
2^+8^-	0·5 ± 0·3	10·3 ± 6·0
2^-8^-	0·8 ± 0·6	6·0 ± 6·6
$CD3^-$	75·7 ± 15·3	62·3 ± 11·4
4^+8^-	0·7 ± 1·0	5·5 ± 3·5
4^+8^+	71·5 ± 15·9	29·4 ± 11·1
4^-8^-	2·5 ± 2·4	23·7 ± 5·8
4^-8^+	1·0 ± 1·7	3·7 ± 5·0

DISCUSSION

In this study, porcine $\alpha\beta$ and $\gamma\delta$ T cells and NK cells have been identified and classified, revealing a remarkable cellular heterogeneity, as defined by surface marker analysis. In particular, some interesting subpopulations which are unique or inaccessible in man and mouse have been identified and their function both in health and disease can now be explored.

Previous identification of porcine T lymphocytes were approached by indirect criteria, such as non-adherence to nylon wool, lack of surface immunoglobulin, expression of CD2, CD4 and CD8, or other physiological properties.[5] Consequently, porcine T lymphocytes was initially resolved into two subsets of conventional T ($CD2^+$) and 'null' T ($CD2^-$ surface immunoglobulin-negative),[20] and then more recently into four subsets defined by CD4 and CD8 ($CD4^+8^-$, $CD4^+8^+$, $CD4^-8^+$ and $CD4^-8^-$).[21] Further attempts to classify porcine T cells more precisely have been frustrated because porcine T-cell subpopulations do not exactly mirror their human or rodent counterparts. As revealed by this work, the CD2- and CD8-bearing cells are heterogeneous and include cells of quite different nature: $\alpha\beta$ and $\gamma\delta$ T cells and NK cells. In particular, given that a high proportion of NK cells are present in young animals, using CD2 or CD8 to identify or prepare T cells may cause overestimation of their proportion. This explains why the low percentage of $\alpha\beta$ T cells in very young pigs has not been realized before. In this study, it is the genuine T-cell markers we used that have provided identification and enumeration of an unexpectedly complex set of lymphocyte subpopulations.

This study also demonstrates that porcine $CD4^+$ (including $CD4^+8^{lo}$) and $CD8^{hi}$ cells are all $\alpha\beta$ cells. Furthermore, the $CD8^+\alpha\beta$ T cells are resolved into two subsets according to their CD8 antigen density, a feature which may reflect functional differences rather than merely quantitative variations in CD8 expression. Indeed, previous studies have demonstrated that the $CD8^{hi}$ and $CD8^{lo}$ are distinguished by CD5 expression and MHC-restricted cytotoxicity.[22] In this study, we found that the $CD4^-8^{lo}$ and $CD4^+8^{lo}$ $\alpha\beta$ T cells, some $\gamma\delta$ T cells and NK cells all expressed CD8 at a similar low level, displaying a similar phenotype ($CD2^+8^{lo}$) except for CD3 and CD4 expression. Therefore, it is possible the $CD4^-8^{lo}$ $\alpha\beta$ T cells form a functionally different subset. In support with this notion, we have recently selected a mAb differentiating $CD8^{hi}$ $\alpha\beta$ T cells from $CD8^{lo}$ cells (H. Yang and R.M. E. Parkhouse, unpublished work). In spite of the previous assumption of $CD4^-8^+$ cells as conventional cytotoxic T cells,[23] therefore, it now appears that the $CD4^-8^+$ cells (even excluding NK cells and $\gamma\delta$ T cells) are too heterogeneous to include a single functional subset.

It is interesting that the CD3 molecule is differentially expressed on different T-cell subsets, in particular, between $CD4^+8^-$ and $CD4^+8^{lo}$ $\alpha\beta$ T cells. It has been postulated that the $CD4^+8^{lo}$ cells represent previously activated cells as they responded to superantigen or second antigen challenge.[5] With this in mind, the lower CD3 expression observed on this cell subset might be associated with previous activation. Similarly, the difference in CD3 expression between $CD8^-$ and $CD8^{lo}$ $\gamma\delta$ T cells also raises the possibility that the $CD8^{lo}$ $\gamma\delta$ T cells may be previously activated cells. This interpretation is supported by previous findings made in other species. For instance, mouse $\gamma\delta$ T cells have been found to acquire CD8 following activation[24] and chicken $CD8^+$ $\gamma\delta$ T cells express the activation marker MHC class II and represent the major $\gamma\delta$ T cells responsive to receptor ligation and exogenous growth factors.[25] Thus, the decreased expression of CD3 on porcine $CD4^+8^{lo}$ $\alpha\beta$ T and $CD8^{lo}$ $\gamma\delta$ T cells might reflect a lower threshold for activation via CD3, which is made possible by the additional presence of costimulating markers such as CD8.

Another striking observation is that the proportion of peripheral blood $\alpha\beta$ T cells was unexpectedly low in young pigs (aged 4 weeks to 4 months) with only 4·7% of the total PBL in the most extreme pig examined. Thus, $\gamma\delta$ T cells comprise the

Table 4. Identification of porcine NK cells as $CD2^+3^-8^{lo}$; cells bearing the indicated markers were selectively depleted from PBL by panning before being used as effector cells in the cytotoxicity assays. ^{51}Cr-labelled K562 cells were used as the targets. Values are mean percentage of specific ^{51}Cr release ± SD (n = 3)

Experiment No.	Phenotype of cells after depletion				
	PBL	$CD2^-$	$CD4^-$	$CD8^-$	$CD3^-$
1	13·5 ± 3·6	5·4 ± 1·2	20·0 ± 5·3	4·6 ± 0·8	59·6 ± 7·3
2	14·0 ± 5·6	4·6 ± 1·8	12·0 ± 7·5	4·4 ± 0·6	69·1 ± 9·2
3	21·6 ± 1·8	4·6 ± 0·7	23·7 ± 1·5	3·4 ± 8·1	73·6 ± 6·6

major T cells in young pigs, in sharp contrast to human and rodents where most T cells are $\alpha\beta$ T cells. The similarly large proportion of $\gamma\delta$ T cells in the large thymocytes is also different to human and rodents where $\gamma\delta$ T thymocytes are present at a much lower frequency.[26–28] It appears, therefore, that the $\gamma\delta$ T cells may play a more important role in young pigs than currently realized. Combined with the high frequency of NK cells, these observations suggest that cellular immunity in the young pigs depends more on innate immunity coupled with $\gamma\delta$ T-cell activity, later to be superseded and assisted by conventional $\alpha\beta$ T cells as the antigenic experience of the animal increases their T-cell repertoire during development. As $\gamma\delta$ T cells also represent the major T-cell subset in some other species, it would be of interest to investigate whether this is a general phenomenon.

The porcine NK cells have been identified in this study as cells with a phenotype of sIg^- $CD2^+3^-8^{lo}$, similar to human NK cells.[29] However, whilst human NK cells are mainly so-called large granular lymphocytes,[29] the size and granular contents (defined by forward and side scatter parameters, respectively) of porcine NK cells were found to be within the range of other lymphocyte subpopulations. In addition, in contrast to the change in the proportion of porcine NK activity-containing cells, the proportion of their human counterpart found in cord blood (19% on average) is not very different from that in adults (15% on average).[29] Whether these dissimilarities have functional implications is unclear.

In summary, this work provides a detailed phenotypic classification of porcine lymphocyte subsets and thus calls for further studies on their immunobiology, in particular, the functions of rare T-cell subsets. Also, it raises some issues which may have implications in other species.

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REFERENCES

1. Sachs D.H. (1994) The pig as a potential xenograft donor. *Vet Immunol Immunopathol* **43**, 185.
2. Binns R.M. & Pabst R. (1988) Lymphoid cell migration and homing in the young pig: alternative immune mechanisms in action. In: Migration and homing of lymphoid cells (ed. A.J. Husband), Vol. 2, p. 137. CRC Press, Boca Raton.
3. Pescovitz M.D., Lunney J.K. & Sachs D.H. (1985) Murine anti-swine T4 and T8 monoclonal antibodies. Distribution and effects on proliferative and cytotoxic T cells. *J Immunol* **134**, 37.
4. Saalmuller A., Reddehase M.J., Buhring H-J., Jonjic S. & Koszionowski U.H. (1987) Simultaneous expression of CD4 and CD8 antigens by a substantial proportion of resting porcine T lymphocytes. *Eur J Immunol* **17**, 1297.
5. Pescovitz M.D., Sakopoulos A.G., Gaddy J.A., Husmann R.T. & Zuckermann F.A. (1994) Porcine peripheral blood $CD4^+$/$CD8^+$ dual expressing T cells. *Vet Immunol Immunopathol* **43**, 53.
6. Duncan I.A., Binns R.M. & Duffus W.P. H. (1989) The null T cell in pig blood is not an NK cell. *Immunology* **68**, 392.
7. Hirt W., Saalmuller A. & Reddehase M.J. (1990) Distinct γ/δ T cell receptors define two subsets of circulating porcine $CD2^-CD4^-CD8^-$ T lymphocytes. *Eur J Immunol* **20**, 265.
8. Saalmuller A., Hirt W. & Reddehase M.J. (1990) Porcine γ/δ T lymphocyte subsets differing in their propensity to home to lymphoid tissue. *Eur J Immunol* **20**, 2343.
9. Mackay C.R. & Hein W.R. (1989) A large proportion of bovine T cells express the $\gamma\delta$ T cell receptor and show a distinct tissue distribution and surface phenotype. *Int Immunol* **5**, 540.
10. Binns R.M., Duncan I.A., Powis S.J., Hutchings A. & Butcher G.W. (1992) Subsets of null and $\gamma\delta$ T-cell $receptor^+$ T lymphocytes in the blood of young pigs identified by specific monoclonal antibodies. *Immunology* **77**, 219.
11. Carr M.M., Howard C.J., Sopp P., Manser J.M. & Parsons K.R. (1994) Expression on porcine $\gamma\delta$ lymphocytes of a phylogenitically conserved surface antigen previously restricted in expression to ruminant $\gamma\delta$ T lymphocytes. *Immunology* **81**, 36.
12. Thome M., Saalmuller A. & Pfaff E. (1993) Molecular cloning of porcine T cell receptor α, β, γ and δ chains using polymerase chain reaction fragments of the constant regions. *Eur J Immunol* **23**, 1005.
13. Yang H., Oura C.A.L., Kirkham P.A. & Parkhouse R.M.E. (1996). Preparation of monoclonal anti-porcine CD3 antibodies and preliminary characterization of porcine T lymphocytes. *Immunology*, in press.
14. Hammerberg C. & Schurig G.G. (1986) Characterization of monoclonal antibodies directed against swine leucocytes. *Vet Immuol Immunopathol* **11**, 107.
15. Pescovitz M.D., Lunney J.K. & Sachs D.H. (1984) Preparation and characterization of monoclonal antibodies reactive with porcine PBL. *J Immuno.* **133**, 368.
16. Jonjic S. & Koszinowski U.H. (1984) Monoclonal antibodies reactive with swine lymphocytes.1. antibodies to membrane structures that define the cytolytic T subset in the swine. *J Immunol* **133**, 647.
17. Yang H. & Binns R.M. (1993) Expression and regulation of the porcine CD44 molecules. *Cell Immunol* **149**, 117.
18. Wysocki L.J. & Sato V.L. (1978) "Panning" for lymphocytes: a method for cell selection. *Proc Natl Acad Sci USA.* **75**, 2844.
19. Yang H., Hutchings A. & Binns R.M. (1993) Preparation and reactivities of anti-porcine CD44 monoclonal antibodies. *Scand J Immunol* **37**, 490.
20. Binns R.M. (1994) The null/$\gamma\delta TCR^+$ T cell family in the pig. *Vet Immunol Immunopathol* **43**, 69.
21. Reddehase M.J., Saalmuller A. & Hirt W. (1991) γ/δ T-lymphocyte subsets in Swine. *Curr Top Microbiol Immunol* **173**, 113.
22. Saalmuller A., Hirt W., Maurer S. & Weiland E. (1994) Discrimination between two subsets of porcine $CD8^+$ cytolytic T lymphocytes by the expression of CD5 antigen. *Immunology* **81**, 578.
23. Saalmuller A. & Bryant J. (1994) Characteristics of porcine T lymphocytes and T-cell lines. *Vet Immunol Immunopathol* **43**, 45.
24. Spetz A-L., Kourilsky P. & Laursson-Sciard E-L. (1991) Induction of CD8 molecules on thymic γ/δ T cells *in vitro* is dependent upon α/β T cells. *Eur J Immunol* **21**, 2755.
25. Kasahara Y., Chen C.H. & Cooper M.D. (1993) Growth requirements for avian $\gamma\delta$ T cells include exogenous cytokines, receptor ligation and *in vivo* priming. *Eur J Immunol* **23**, 2230.
26. Allison J.P. & Havran W.L. (1991). The immunobiology of T cells with invariant $\gamma\delta$ antigen receptors. *Annu Rev Immunol* **9**, 679.
27. Sato K., Ohtsuka K., Watanabe H., Asakura H. & Abo T. (1993) Detailed characterization of $\gamma\delta$T cells within the organs in mice: classification into three groups. *Immunology* **80**, 380
28. Haas W., Pereira P. & Tonegawa S. (1993) Gamma/delta cells. *Annu Rev Immunol* **11**, 637.
29. Trinchieri G. (1989) Biology of natural killer cells. *Adv Immunol* **47**, 187.

