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Functional and phenotypic analysis of porcine peripheral blood CD4/CD8 double-positive T cells

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SUMMARY

Functional and phenotypic properties of porcine peripheral blood CD4/CD8 double-positive (DP) lymphocytes were examined. In cross-sectional and longitudinal studies involving a total of 103 pigs, this lymphocyte population was found to increase gradually in proportion with age, comprising <2% of the total peripheral blood lymphocyte pool in 1-week-old swine and reaching 30–55% by 3 years of age. CD4/CD8 DP lymphocytes were able to proliferate in response to stimulation with recall viral antigen. Furthermore, these cells mostly expressed high levels of the surface antigen recognized by monoclonal antibody (mAb) 4B4 ($4B4^{hi}$), which is specific for the human $\beta 1$ integrin. The $CD4^{+}4B4^{hi}$ lymphocytes from pseudorabies virus-immune swine, proliferated in response to stimulation with the homologous virus, while $CD4^{+}4B4^{lo}$ lymphocytes did not. Stimulation of CD4 single-positive (SP) cells with recall viral antigen, but not with mitogen, resulted in the generation of lymphoblasts which were predominantly of CD4/CD8 DP phenotype, suggesting a role for recall antigen in the generation of this lymphocyte subset. More than half of the $CD4^{+}$ lymphocytes from palatine tonsils of 6-month-old swine were CD4/CD8 DP, while in the lymph nodes CD4/CD8 DP cells accounted for only one-third or less of $CD4^{+}$ cells. In contrast, CD4/CD8 DP lymphocytes were absent from the palatine tonsils of 3-day-old swine, which only contained CD4 SP cells. Together, these results indicate that porcine CD4/CD8 DP lymphocytes, exhibit properties of mature antigen-experienced cells, and are inducible by stimulation with recall antigen. These data are consistent with the hypothesis that this population in swine includes memory/effector T cells.

INTRODUCTION

In normal adult pigs, a substantial proportion (10–60%) of peripheral lymphocytes can simultaneously express CD4 and CD8 coreceptors.[1–4] Because of the abundance of CD4/CD8 double-positive (DP) lymphocytes in porcine peripheral blood, it is important to understand the biological significance of this unconventional lymphocyte population. Although CD4/CD8 DP lymphocytes are rare (<3%) in human peripheral blood,[5–7] higher proportions (>15%) can be detected in patients suffering from some types of neoplasia, infectious mononucleosis, and occasionally in healthy individuals.[8] CD4/CD8 DP cells have also been found among sites of strong inflammatory response such as the joint fluid of rheumatoid arthritis patients[9] and among lymphocytes infiltrating human[10] and porcine[11] renal allografts. In addition, it has become increasingly evident that CD4/CD8 DP cells are a normal component of the intestinal intraepithelial lymphocyte (iIEL) population of both mice and rats.[12–14] In swine, the peripheral blood CD4/CD8 DP lymphocyte subset is present in addition to the typical CD4 single-positive (SP) and CD8 SP subsets which exhibit typical helper and cytotoxic functions.[15,16] Porcine CD4/CD8 DP cells are also normally present in the spleen, lymph nodes and tonsils of adult pigs.[3]

Two possible sources of human and porcine CD4/CD8 DP lymphocytes have been proposed. First, that these cells represent immature precursors emigrated from the thymus without prior differentiation. Second, that these cells represent $CD4^{+}$ T-helper lymphocytes that have acquired the CD8 antigen upon prior sensitization and retained it after reversion to small lymphocytes.[3,7] On the one hand, the expression, or lack thereof, of certain cell surface molecules by porcine peripheral blood CD4/CD8 DP cells makes the first alternative unlikely. On the basis of the expression of the 8/1 antigen (a marker for resting porcine T cells that is down-regulated after activation), the size of the cells, the lack of expression of CD1 and high CD5 expression, porcine peripheral blood CD4/CD8

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Abbreviations: DN, double-negative; DP, double-positive; iIEL, intestinal intraepithelial lymphocytes; PrV, pseudorabies virus; SEB, staphylococcal enterotoxin B; SP, single-positive; and SPV, swine pox virus.

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DP T cells are thought to belong to a mature resting population of lymphocytes.[3,17–20] While CD4/CD8 DP human cells have been shown to express CD3 at levels characteriztic of mature T cells,[7] similar evidence for porcine CD4/CD8 DP lymphocytes does not exist since antibodies specific for porcine CD3 and T-cell receptors (TCR) are not available.[21] However, these cells are considered to be T cells by their non-adherence to nylon wool, the presence of CD2 antigen, and the absence of surface immunoglobulin.[1–3] Furthermore, sorted populations of porcine CD4/CD8 DP lymphocytes proliferate in response to stimulation with the superantigen Staphylococcal enterotoxin B (SEB), indicating that they express some type of TCR and are functionally mature.[4] On the other hand, indirect evidence suggesting that a phenotypic switch among porcine T lymphocytes from CD4 SP cells to CD4/CD8 DP may occur is the observation that a predominant proportion of swine lymphoblasts generated in an *in vitro* response to alloantigen,[3] viral,[4] or parasite[22] antigens is of CD4/CD8 DP phenotype. Recently, in a model system of inflammatory bowel disease, mature CD4 SP cells were shown to have been induced to express CD8α *in vivo*, and become CD4/CD8 DP cells.[23] Similar to what had been shown previously for human and porcine CD4/CD8 DP peripheral blood lymphocytes,[3,7,22] the phenotype of the CD4/CD8 DP iIEL generated in this bowel disease model was stable *in vitro*.[23] Both human peripheral blood CD4/CD8 DP lymphocytes and murine CD4/CD8 DP iIEL express exclusively the $\alpha\beta$ TCR and have helper function.[7,10,24] In spite of the abundance of CD4/CD8 DP in the peripheral blood of swine, the functional capacity and possible role of this lymphocyte subset have not been examined.

The present study was initiated to delineate some of the properties of porcine peripheral blood CD4/CD8 DP lymphocytes. The properties studied include the influence of age on the presence of this lymphocyte population, the identification of a cell surface phenotypic marker that distinguishes between naive and memory/effector T cells, the role of antigen in the generation of these cells, and the distribution of CD4/CD8 DP lymphocytes within selected secondary lymphoid organs. Functional properties were analysed by measuring a virus-specific secondary lymphoproliferative response of sorted populations of peripheral blood mononuclear cells from swine previously immunized against pseudorabies virus (PrV), a herpesvirus of swine. The resultant data indicate that cells responsive to recall viral antigen are present among both CD4/CD8 DP and CD4 SP lymphocytes. The results are consistent with the postulate that the porcine peripheral blood CD4/CD8 DP lymphocyte population includes mature lymphocytes which exhibit properties characteriztic of memory/effector cells and that cells with this phenotype are inducible by stimulation with recall-antigen.

MATERIALS AND METHODS

Animals and tissues

Cross-bred domestic swine (Yorkshire × Landrace × Duroc) of various ages from the University of Illinois (U of I) College of Veterinary Medicine (CVM) Research Farm were utilized as blood donors for the aging study. Animals are certified to be free of most swine diseases including pseudorabies virus (PrV) and swine pox virus (SPV). All animal procedures were approved by the office of laboratory animal care based on federal guidelines. Blood samples were also collected from cross-bred (Camborough × F26) F_1 pigs at 6 months of age from a local farm. Major histocompatibility complex-homozygous miniature swine of $SLA^{c/c}$ haplotype[25] were purchased from either the University of Iowa swine breeding herd, or from the Tufts University swine herd. These animals were negative by serological and cellular assay to both PrV and SPV at the time of purchase. For the analysis of cellular immune responses to PrV or SPV, cross-bred domestic swine from the U of I herd or miniature swine at 6 months of age were transferred to an isolation facility and vaccinated against PrV via intramuscular (i.m.) injection with a commercial PrV live-modified vaccine (Tolvid, Upjohn, Kalamazoo, MI) or infected by scarification with a wild-type strain of SPV (kindly provided by Dr Deoki Tripathy, U of I). Tonsils and peripheral lymph nodes were collected from healthy market-weight pigs (5–6 months of age) immediately after slaughter at the U of I Meat Science Laboratory. The same tissues were obtained from healthy cross-bred newborn swine purchased from the U of I CVM Research Farm. Lymphocyte suspensions from these organs were obtained by cutting the tissues into small pieces and gently sieving through a fine metal mesh. Dead cells were then removed by centrifugation over a cushion of Ficoll–Hypaque 1077 from Sigma (St. Louis, MO).

Monoclonal antibodies and cytokines

Monoclonal antibodies (mAb) specific for porcine CD4 (74-12-4, mouse IgG2bκ), and CD8 (76-2-11, mouse IgG2aκ) were purchased from the American Type Culture Collection (ATCC; Rockville, MD). The specificities of these mAb have been described previously[26] and were further characterized during the First International Swine CD Workshop.[21] The RD1-labelled human β1 integrin-specific mAb 4B4[27] was purchased from Coulter (Hialeah, FL). Unlabelled 4B4 (anti-human VLA-β1); 8F2 (anti-human VLA-α4); 2H6 (anti-human VLA-α5); and L25 (anti-human VLA-α4)[28] were kindly provided by Dr Chikao Morimoto, Dana-Farber Cancer Institute, Boston, MA. A mAb to porcine CD45 (K252.1E4)[29] was kindly provided by Dr Chris Stokes, Bristol, UK. Monoclonal antibodies specific for porcine CD4 and CD8 were purified by passage through a protein A–Sepharose column using standard procedures according to the mAb isotype. The eluates were dialysed against phosphate buffered saline (PBS) and adjusted to a concentration of 2 mg/ml. CD4 and CD8 mAb were conjugated to biotin-*n*-hydroxy succinimide ester (Calbiochem) using the method of Jackson *et al.*[30] Human recombinant interleukin-2 (IL-2) was purchased from R&D Systems, Minneapolis, MN.

Culture medium

HEPES-buffered RPMI-1640 (Gibco, Grand Island, NY), was supplemented with 2×10^{-5} M 2-mercaptoethanol (Sigma, St. Louis, MO), 2 mM sodium pyruvate (Gibco), 2 mM L-glutamine (Gibco), 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma), 1 μg/ml Gentamycin (Sigma), 10% non-essential aminoacids (Gibco), and 2% fetal porcine serum (Gibco).

Cell isolation and lymphoproliferation assay

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized venous blood using Ficoll–Hypaque 1077 (Sigma) gradient centrifugation. Virus-specific memory/effector

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T cells, were isolated from PBMC within 1 to 8 months after immunization with PrV or SPV. PBMC were cultured at 5×10^5/well in sterile 96-well round bottom plates in a 200 μl volume of RPMI culture medium, and were stimulated with viral antigen for 4 days in a humidified incubator with 5% CO_2 at 37°. Viral antigen consisted of sucrose gradient-purified ultraviolet (UV)-inactivated PrV strain Kaplan [PrV(Ka)] which had been grown in pig kidney (PK) cells and prepared as previously described,[15] or SPV-infected PK cell lysates harvested at the time of maximal cytopathogenic effect (usually by 5 days postinfection). Pretests showed that the optimal stimulating dose of PrV was 1–10 μg/ml and for SPV it was a 1 : 100 dilution of the SPV-infected cell lysate (10^4 PFU/ml final dose). All assays were done with these two doses of PrV antigen and the response to the optimal dose was presented. Herpes simplex virus-2 (HSV-2) stocks (kindly provided by Dr Gail Scherba, University of Illinois), were grown in HeLa cells, inactivated by UV radiation and used at a 1 : 100 dilution. Mock-infected PK cell culture lysates (1 : 100 dilution) were used as negative controls for mock stimulation. Positive response controls consisted of stimulation with the T-cell mitogen phytohaemagglutinin (PHA) (5 μg/ml) (Gibco), or SEB (Sigma) at 1 μg/ml. The lymphoproliferative response was measured by the addition of 50 μl/well of culture medium containing 1 μCi of [^{3}H]thymidine (New England Nuclear, specific activity 52 Ci/mmol) during the last 12–16 hr of culture. Cells were harvested onto glass fibre filter paper with a PHD cell harvester (Cambridge Technologies, Boston, MA), and the incorporated radioactivity was measured by liquid scintillation spectrophotometry and used as a measure of lymphocyte proliferation. Results are expressed as mean counts per minute (c.p.m.) ± standard deviation of triplicate cultures without background subtraction. Background incorporation ranged from 300 to 900 c.p.m.

Two- and three-colour analysis and fluorescent-activated cell sorting of T cells

Peripheral blood $CD4^+CD8^+$, $CD4^+CD8^-$ and $CD4^-CD8^+$ cells were isolated by two-colour FACS with an Epics 752 Flow Cytometer (Coulter) equipped with an Argon ion laser. Briefly, 6–8 Eppendorf tubes containing suspensions of glass-adherent cell-depleted PBMC (10^7/tube), in sterile Flow PBS (0·1 M PBS, pH 7·2, supplemented with 0·5% bovine serum albumin), were reacted with 10 μg/tube of anti-CD8 mAb in a 100 μl volume. After a 30 min incubation on ice, cells were washed with Flow PBS and reacted with fluorescein isothiocyanate (FITC)-labelled goat anti-mouse immunoglobulin $F(ab')_2$ antibody (Zymed, San Francisco, CA) for 30 min. After washing, free binding sites were blocked by incubating the cells with 30 μl of purified mouse immunoglobulin (100 μg/ml, Zymed). Cells were then reacted with 10 μg of biotin-conjugated anti-CD4 mAb followed, after washing, by 10 μg of streptavidin–phycoerythrin (Zymed). After a final wash, cells were suspended at 10^7/ml in Flow PBS and sorted by gating on small lymphocytes based on their forward angle and 90° angle light-scatter. Sort windows to separate between $CD4^+CD8^+$ (double positive) $CD4^+CD8^-$ (single positive) cells were set very conservatively to select $CD4^+$ cells which were the two-thirds most $CD8^-$ or the two-thirds most $CD8^+$. For all experiments shown, cytofluorometric analysis of sorted cells showed that they were > 94% of the sorted phenotype. Stained unsorted, and unstained cells serve as controls of the possible effect of treatment on the response to stimulation. Analytical two-colour cytofluorometry for CD4 and CD8 expression was done as above except that the reagents were scaled down to stain 10^6 cells/tube. For the sorting of $CD4^+4B4^{hi}$ and $CD4^+4B4^{lo}$ cell subsets, PBMC were stained sequentially with anti-CD4 mAb 74-12-4 followed by FITC-labelled goat anti-mouse immunoglobulin $F(ab')_2$ antibody (Zymed) for 30 min each. After washing, free binding sites were blocked by incubating the cells with 30 μl of purified mouse immunoglobulin (100 μg/ml, Zymed) for 20 min followed by the addition of RD1-labelled anti-human CD29 mAb 4B4 (Coulter). The stained cells were sorted as above into $CD4^+$ cells which were either $4B4^{hi}$ (the two-thirds 4B4 brightest cells) or $4B4^{lo}$ (the two-thirds 4B4 dullest cells). The purity of the sorted cells was determined to be > 95% by analysis of the sorted cells.

Three-colour staining of the expression of CD4, CD8 and 4B4 was obtained by reacting PBMC in the following labeling sequence: (1) incubation with anti-CD8 mAb 76-2-11; (2) wash twice; (3) incubation with FITC-conjugated goat anti-mouse IgM, IgG $F(ab')_2$ (Zymed); (3) wash twice; (4) saturate free binding sites of the FITC-conjugated antibody with mouse IgG (200 μg/ml) (Zymed); (5) incubate with biotin-conjugated anti-CD4 mAb 74-12-4; (6) wash twice; (7) staining with streptavidin-RED670 (Gibco); (8) wash twice; (9) stain with RD1-labelled anti-CD29 mAb 4B4 (Coulter). All incubations were done for 30 min on ice in a 50 μl volume. Three-colour fluorescence analysis was performed in an EPICS 752 flow cytometer (Coulter Electronics) equipped with an argon ion laser. Cells labelled with only one dye were used to monitor spectral emission overlap. Correction for fluorescein spillover into the orange (RD1) channel was corrected by electronic compensation by subtracting 10–15% of the fluorescein signal. Staining with either a biotin-conjugated murine mAb of irrelevant specificity followed by streptavidin-RED670 was used to discriminate between red positive and negative signal. Staining with an irrelevant primary mAb followed by goat anti-mouse IgM and IgG $F(ab')_2$, or an irrelevant specificity phycoerythrin (PE)-labelled mouse immunoglobulin of matched isotype as mAb 4B4, were used to discriminate between the green and orange specific signals, respectively. Data were collected for 20 000 cells using the MDADS Coulter data acquisition software in list mode. The data were analysed utilizing ELITE software from Coulter. In the forward versus right angle light scatter histogram, electronic gates were set so as to include only small lymphocytes. Fluorescence intensities in the red (Red670) and green (FITC) fluorescence axes were expressed on a 3 log scale. Orange (RD1) fluorescence intensity was expressed on a 256-channel scale which represents 3 logs of fluorescence intensity.

Culture of lymphocytes purified by FACS and culture of short-term cell line

FACS-purified lymphocytes were cultured in 96-well round bottom plates at 2×10^5 cells per well. As a source of antigen-presenting cells (APC), autologous glass-adherent cells were isolated as previously described.[16] Glass-adherent cells at 5×10^6 cells/ml were treated with mitomycin C (50 μg/ml) (Sigma) for 1 hr at 37° protected from light. Mitomycin C-treated glass-adherent cells were added to the cultures at a

concentration of $2{\cdot}5 \times 10^4$ cells per well. Cells isolated by this procedure were < 5% CD4 and/or CD8 and did not respond to stimulation with PrV by either proliferation or IL-2. A short-term cell line was maintained for 10 weeks starting from $CD4^+CD8^-$ lymphocytes from one of the sorting experiments. This cell line was kept in 96-well round bottom plates at a density of 5×10^5 cells/well at the beginning of each of several cycles of stimulation which consisted of the addition every 14 days of viral antigen (sucrose gradient-purified PrV at 10 μg/ml) in the presence of mitomycin C-treated autologous glass-adherent cells ($2{\cdot}5 \times 10^4$ cells/well) and the addition every 4 days of 10 U/ml of human recombinant IL-2 (R&D Systems, Minneapolis, MN).

Lactoperoxidase-catalysed cell surface iodination and immunoprecipitation

Lymphocyte surface antigens were labelled with ^{125}I using a lactoperoxidase catalysed reaction.[26] Briefly, washed, > 97% viable porcine PBMC (5×10^7 cells) were suspended in 200 μl of PBS at room temperature. Fifty microlitres of lactoperoxidase (0·4 mg/ml, type V, Sigma) were added to the cell suspension followed by 1 mCi of ^{125}I (New England Nuclear, Boston, MA) at room temperature. Three 50 μl aliquots of 0·03% H_2O_2 were added at 5-min intervals. The reaction was stopped after 15 min by 40-fold dilution and three times washing in ice-cold PBS containing 0·02% (w/v) NaN_3 and 5 mM KI. Cells were lysed in 1 ml of 10 mM Tris–HCl, pH 8·2, 1% (w/v) nonidet P-40 (NP-40), 150 mM NaCl, 1 mM EDTA, 1% (w/v) haemoglobin, containing 1 mM phenylmethylsulphonyl fluoride (PMSF), 1 mM iodoacetamide and 20 U of aprotinin, for 60 min at 4°. Nuclei and debris were removed from the lysate by centrifugation at 6000 *g* for 10 min. Lymphocyte lysates were precleared with 200 μl of a 50% (v/v) slurry of protein A–Sepharose (Pharmacia, Piscataway, NJ). Samples of the lysate (2×10^6 c.p.m.) were reacted with 5 μl of mAb K252.1E4 tissue culture supernatant or with 2 μl of mAb 4B4, L25, 8F2, or 2H5 ascites and incubated for 2 hr at 4°. Fifty microlitres of a 50% (v/v) suspension of protein A–Sepharose in lysis buffer was added, and after an additional 2 hr incubation at 4°, the Sepharose beads were washed sequentially once in ice-cold lysis buffer, twice in TE buffer and once in 50 mM Tris pH 6·5. The washed pellet was suspended in sample buffer (Novex, San Diego, CA), boiled for 5 min in the presence of 5% 2-mercaptoethanol, and resolved in a 6% Tris–Glycine sodium dodecylsulphate polyacrylamide gel. The resolved bands were visualized by autoradiography of dried gels with XRO-MAT film (Kodak, Rochester, NY). Molecular weights (MW) were estimated based on prestained MW standard (Novex, San Diego, CA).

Statistical analysis

Linear regression, correlation analysis, two-tailed *t*-test and analysis of variance (ANOVA) were done with STATVIEW software (Abacus Concepts Inc., Berkeley, CA).

RESULTS

The proportion of peripheral blood CD4/CD8 double positive T cells in swine increases with age

In agreement with previous studies[2,3] peripheral blood lymphocytes obtained from conventionally reared 5–6-month-old pigs had a considerable proportion (11·3 ± 2·2%, $n = 15$) of CD4/CD8 DP cells (Table 1, PIC pigs). To extend these observations, we examined the effect of age on the presence of this cell population in cross-sectional and longitudinal studies. In the cross-sectional study, the proportion of peripheral blood CD4 SP, CD8 SP, and CD4/CD8 DP cells of the total lymphocyte population, was evaluated in blood samples obtained from randomly selected cross-bred pigs ranging in age from 0·1 to 54 months (Table 1, U of I pigs). The CD4/CD8 DP lymphocyte subset represented < 2% of the total lymphocyte population in 1-week-old swine, 4·6% in 1-month-old, and 5% in 2- and 3-month-old swine. The proportion continued to rise gradually with increasing age and constituted on average 10% of the total blood lymphocytes in pigs aged 5 months, 15% in pigs aged 12–20 months, 23% in those aged 25–40 months, and 35% in swine aged 45–54 months. When these variables were analysed for their possible association, a strong positive correlation ($R^2 = 0{\cdot}91$) between age and the percentage of CD4/CD8 DP lymphocytes was found ($P = 0{\cdot}006$). No significant age-associated changes in the proportion of CD4 SP lymphocytes were observed among the ages examined. The proportion of CD8 SP lymphocytes tended to increase with age ($R^2 = 0{\cdot}65$; $P = 0{\cdot}05$).

To determine the effect of age on the development of T-cell subsets in the same animals and in a more homogeneous population, a longitudinal study was conducted in major histocompatibility complex (MHC)-homozygous miniature pigs. Initially, two NIH miniature pigs were examined once at 3 weeks of age. The proportion of CD4/CD8 DP cells in these animals was 4% of total blood lymphocytes. Subsequently, groups of miniature pigs of $SLA^{c/c}$ haplotype aged 7 months (six animals) or 27 months (two animals) were examined six times over a 26-month period (Table 1, NIH pigs). The younger group of miniature pigs had a gradual increase in the proportion of CD4/CD8 DP cells among the total lymphocyte population. The average proportion of DP cells more than doubled from an average of 7% at 7 months of age, to 15% at 18 and 21 months, continuing to increase to 19% by 26 months, and reaching 24% by 32 months of age. In these animals, the absolute number of CD4/CD8 DP lymphocytes in the peripheral blood was 1×10^6/ml of blood at 9 months of age, increased to $1{\cdot}4 \times 10^6$/ml at 18 months of age, and increased further to $2{\cdot}3 \times 10^6$/ml by 32 months of age ($P < 0{\cdot}01$). In the older NIH miniature swine, which at the time of the first sampling were already 27 months of age, the percentage of CD4/CD8 DP within the total lymphocyte population was fairly high (30%), reaching 37% by 40 months of age, remaining at the same proportion 3 months later, but increasing to an average of 55% of total lymphocytes by 46 months of age, remaining at that proportion 6 months later. The absolute number of CD4/CD8 DP cells in these two older swine was $2{\cdot}4 \times 10^6$/ml of blood at 27 months of age, and increased markedly to 7×10^6/ml of blood by 52 months of age ($P < 0{\cdot}01$). Similar to the cross-sectional study, these data yielded a strong correlation ($R^2 = 0{\cdot}9$) between age and the proportion of CD4/CD8 DP cells ($P < 0{\cdot}0005$). Of note was the finding that the slope and intercept obtained from the correlation analyses between age and the proportion of CD4/CD8 DP cells in both the cross-sectional and longitudinal studies were not different. This indicates a remarkable

Table 1. Analysis of the proportion of CD4/CD8 double positive, CD4 single positive and CD8 single positive lymphocytes in the peripheral blood of pigs as a function of age

Breed*	Age (months)	n†	Cell population (% ± SD)‡		
			$CD4^+CD8^-$	$CD4^+CD8^+$	$CD4^-CD8^+$**
PIC	6	15	26·3 ± 4·0	11·3 ± 2·2	14·1 ± 2·6
U of I	0·1	5	17·4 ± 3·5	1·8 ± 0·7§	11·0 ± 1·6
U of I	1	7	23·4 ± 2·6	4·6 ± 1·3§	26·0 ± 7·2
U of I	2	16	20·1 ± 2·7	5·0 ± 2·2§	24·5 ± 11
U of I	3	22	21·6 ± 4·5	5·3 ± 2·2§	22·6 ± 4·0
U of I	5	7	20·9 ± 2·6	10·0 ± 1·8§	26·5 ± 2·7
U of I	12–20	9	15·8 ± 3·1	15·2 ± 3·3§	38·6 ± 7·3
U of I	25–40	7	17·8 ± 4·5	23·0 ± 2·8§	35·0 ± 6·2
U of I	45–54	5	11·3 ± 2·7	34·5 ± 6·0§	40·4 ± 5·0
NIH	0·75	2	37·5 ± 4·7	3·5 ± 0·5	17·3 ± 6·0
NIH	7	6††	22·4 ± 2·3	7·4 ± 1·4¶	41·5 ± 5·1
NIH	9	6††	22·5 ± 2·5	8·9 ± 1·4¶	38·0 ± 2·4
			(2·4 ± 0·3)§§¶¶	*(1·0 ± 0·2)*§§***	*(4·0 ± 0·3)*§§
NIH	18	6††	17·3 ± 2·4	15·4 ± 2·2¶	36·1 ± 5·1
			(1·6 ± 0·4)§§¶¶	*(1·4 ± 0·4)*§§***	*(3·6 ± 0·8)*§§
NIH	21	6††	16·0 ± 2·2	15·1 ± 1·9¶	35·5 ± 2·1
NIH	26	4††	13·2 ± 1·9	19·4 ± 1·5¶	33·2 ± 3·2
NIH	32	5††	9·7 ± 1·3	24·3 ± 1·7¶	41·2 ± 1·9
			(0·9 ± 0·1)§§¶¶	*(2·3 ± 0·5)*§§***	*(3·9 ± 0·4)*§§
NIH	27	2‡‡	9·5 ± 2·4	30·5 ± 3·1¶	26·1 ± 4·6
			(0·8 ± 0·3)§§	*(2·4 ± 0·5)*§§†††	*(2·1 ± 0·7)*§§
NIH	30	2‡‡	9·5 ± 2·1	29·9 ± 3·9¶	24·0 ± 5·7
NIH	40	2‡‡	7·2 ± 2·4	37·1 ± 3·2¶	25·0 ± 5·9
NIH	43	2‡‡	7·1 ± 2·1	37·3 ± 5·2¶	22·0 ± 4·2
NIH	46	2‡‡	7·5 ± 0·3	51·8 ± 4·0¶	21·4 ± 4·4
NIH	52	2‡‡	4·0 ± 1·3	51·6 ± 8·0¶	26·4 ± 5·0
			(0·5 ± 0·1)§§	*(7·0 ± 1)*§§†††	*(3·6 ± 0·7)*§§

*U of I, College of Veterinary Medicine SPF herd, cross-bred pigs Yorkshire × Duroc × Landrace; PIC, Pig Improvement Company cross-bred Camborough × Line 26 F-1s pigs; NIH, National Institutes of Health minipigs of $SLA^{c/c}$ haplotype.

† Number of pigs per age.

‡ Peripheral blood lymphocytes were analysed by two-colour cytofluorometry for the expression of CD4 and CD8 antigens as described in the Materials and Methods.

§ Correlation ($R^2 = 0·91$) between age and percentage of $CD4^+CD8^+$ cells ($P = 0·006$).

¶ Correlation ($R^2 = 0·9$) between age and percentage of $CD4^+CD8^+$ cells ($P = 0·0003$).

** Percentage includes $CD4^-CD8^{high}$ and $CD4^-CD8^{low}$ cell populations.

†† The same group of pigs were tested at 7, 9, 18, 21, 26 and 32 months of age.

‡‡ The same two pigs were tested at 27, 30, 40, 43, 46 and 52 months of age.

§§ Values represent absolute cell number (mean ± SEM × 10^{-6})/ml of blood.

¶¶ Comparison of CD4 SP absolute cell number of the same animals at 9 or 18 months of age versus 32 months of age by two-tailed Student's *t*-test ($P < 0·001$).

*** Comparison of CD4/CD8 DP absolute cell number of the same animals at 9 or 18 months of age versus 32 months of age by two-tailed Student's *t*-test ($P < 0·01$).

††† Comparison of CD4/CD8 DP absolute cell number of the same animals at 27 versus 52 months of age by two-tailed Student's *t*-test ($P < 0·01$).

similarity between these two groups of pigs in their rate of increase of the CD4/CD8 DP lymphocyte population with age, as well as in the percentage of these cells at different ages. A strong inverse correlation ($R^2 = 0·93$) between a decreasing proportion of CD4 SP and increasing age ($P = 0·0001$), concomitant with the increase in the proportion of CD4/CD8 DP cells, was also apparent in the miniature swine. The absolute number of CD4 SP cells, which was 2·4 × 10^6/ml at 9 months of age, decreased by more than 50% to 0·9 × 10^6/ml by 32 months ($P < 0·01$). A similar decrease of CD4 SP cells was not seen in the cross-sectional study with the outbred pigs. There was also a trend towards a decrease in the proportion of CD8 SP lymphocytes with increasing age, although it was not statistically significant.

Table 2. Proliferative responses of unfractionated porcine peripheral blood mononuclear cells and CD4 single positive or CD4/CD8 double positive lymphocytes to mitogen, superantigen or viral antigen stimulation*

Experiment Number/ Pig No.†	Immune Status versus PrV	Stimulus	[^{3}H]thymidine incorporation (c.p.m. × 10^{-3} ± SD)			
			$CD4^+CD8^+$	$CD4^+CD8^-$	Stained unsorted	Unstained unsorted
1/7971	Naive	Mock	0·1 ± 0·02	0·1 ± 0·02	0·2 ± 0·02	0·3 ± 0·04
		PrV	0·1 ± 0·01	0·1 ± 0·01	0·2 ± 0·01	0·4 ± 0·1
		PHA	11·0 ± 1·0	125·0 ± 9·0§	133·4 ± 13	156·6 ± 19
2/7052	Immune	Mock	0·1 ± 0·01	0·1 ± 0·01	1·4 ± 0·1	1·9 ± 0·2
		PrV	14·0 ± 2·4	16·2 ± 1·9	20·3 ± 2·0	17·5 ± 1·8
		PHA	18·0 ± 2·1	89·5 ± 4·9§	101·2 ± 2·3	148·4 ± 4·7
3/7050	Immune	Mock	0·2 ± 0·02	0·05 ± 0·01	0·6 ± 0·1	0·4 ± 0·1
		PrV	14·0 ± 1·4	13·5 ± 1·3	14·6 ± 1·5	15·4 ± 1·4
		PHA	62·1 ± 6·1	90·3 ± 7·9	68·2 ± 7·5	67·2 ± 6·9
4/7055	Immune	Mock	0·3 ± 0·01	0·2 ± 0·0	2·3 ± 0·3	ND
		PrV	14·2 ± 1·5‡	5·3 ± 0·5	14·3 ± 1·3	ND
		PHA	15·3 ± 1·8	34·2 ± 5·7	210·2 ± 10	ND
		HSV-2	2·6 ± 0·8	0·6 ± 0·03	2·7 ± 0·5	ND
		SEB	13·5 ± 1·0	37 ± 2·5§	147·6 ± 5·1	ND
5/7052	Immune	Mock	0·1 ± 0·01	0·2 ± 0·05	1·8 ± 0·3	1·9 ± 0·1
		PrV	6·0 ± 1·5	5·4 ± 0·5	22 ± 0·8	19·2 ± 1·7
		PHA	29·1 ± 4·1	101·1 ± 3·7§	154 ± 3·6	135·4 ± 9·9
		HSV-2	0·6 ± 0·3	0·1 ± 0·04	1·5 ± 0·8	1·9 ± 0·9
6/7050	Immune	Mock	0·1 ± 0·01	0·1 ± 0·01	1·8 ± 0·03	1·9 ± 0·02
		PrV	7·4 ± 0·9	4·6 ± 0·5	12·4 ± 1·4	10·3 ± 1·7
		PHA	18·5 ± 1·4	38·6 ± 1·5§	142·3 ± 2·7	111·7 ± 9·6
7/165	Immune	Mock	0·2 ± 0·1	0·1 ± 0·03	ND	0·4 ± 0·4
		PrV	11·2 ± 1·5	8·1 ± 0·4	ND	16·3 ± 0·9
		SPV	0·3 ± 0·1	0·1 ± 0·01	ND	0·5 ± 0·06
		SEB	16·5 ± 0·9	19·4 ± 0·9	ND	31·5 ± 1·9
8/12	Naive/SPV immune	Mock	ND	ND	ND	0·7 ± 0·02
		PrV	ND	ND	ND	0·9 ± 0·31
		SPV	ND	ND	ND	43·6 ± 2·1

*Populations of unfractionated peripheral blood mononuclear (5×10^5 cells/well) and FACS sorted $CD4^+CD8^+$ or $CD4^+CD8^-$ cells (2×10^5 cells/well), isolated from naive or pseudorabies virus immune swine, were stimulated with PHA, SEB or viral antigen (PrV, SPV or HSV-2) for 4 days. Autologous mitomycin-C-treated glass-adherent cells ($2{\cdot}5 \times 10^4$) were added to sorted cell populations. Proliferation was measured by [^{3}H]thymidine incorporation during the final 16 hr of culture. In all of these independent experiments purity of sorted cells was > 94%. Values are expressed as mean c.p.m. ± standard deviation of triplicate samples.

†All pigs were 8–18 months old cross-bred domestic swine except pig 165, which was a NIH miniature swine of the $SLA^{c/c}$ haplotype.

‡$P < 0{\cdot}05$ compared with response of $CD4^+CD8^-$ cells to same stimulus.

§$P < 0{\cdot}05$ compared with response of $CD4^+CD8^+$ cells to same stimulus.

ND, not done.

CD4/CD8 DP and CD4 SP T cells are responsive to recall viral antigen

The gradual increase in the proportion of CD4/CD8 DP lymphocyte subpopulations with age provided support for the hypothesis that these cells might include memory T cells. To test this hypothesis directly, the ability of electronically sorted CD4/CD8 DP, CD4 SP and CD8 SP peripheral blood lymphocytes to mediate a secondary response to viral antigen was investigated. We utilized a viral antigen-specific system in which PBMC from pigs immunized previously against PrV, proliferated in response to stimulation with the homologous virus. This response was virus dose-dependent, being maximal at a dose of 10 μg/ml of viral protein, and peaked by the 3rd day of culture (data not shown). Because of the coexpression of CD4 and CD8 on the population of interest and the presence of both CD4 SP and CD8 SP cells among the starting PBMC population, we had to use positive selection procedures, i.e. electronic cell sorting, to purify CD4/CD8 DP cells. At the beginning of the study we determined that the staining of the cells with mAb against CD4 and CD8 did not have an affect on their ability to respond to stimulation with either recall viral

antigen, superantigen or mitogen as compared to untreated cells in the 4-day proliferation assay (data not shown). Separate experiments demonstrated that antibody-free CD4 and CD8 molecules were present on stained cells after overnight culture (data not shown). Apparently, CD4 and CD8 molecules are either freed from the antibody, or new molecules are expressed after a few hours in culture. In contrast, a blocking effect was observed when excess antibody against CD4 was present in the culture medium throughout the culture period (data not shown). Cytofluorometric analysis of sorted CD4/CD8 DP and CD4 SP lymphocytes showed their purity to be greater than 94% in all experiments.

Both CD4 SP and CD4/CD8 DP cell populations when isolated from PrV-immune pigs were capable of responding with equal intensity to stimulation with identical doses of recall viral antigen (10 μg/ml) in all (Table 2, Experiments 2–6) but one of six experiments in which the CD4/CD8 DP gave a significantly higher response (Table 2, experiment 4). In contrast, in five out of six experiments the magnitude of the proliferative response of CD4 SP cells was 2–11 times higher ($P < 0{\cdot}05$) than that of CD4/CD8 DP cells to the T-cell mitogen PHA (Table 2, experiments 1, 2, 4, 5 and 6). The fact that the [^{3}H]thymidine incorporation by PHA or SEB-stimulated cultures of unsorted cells was 1·5–3 times larger than that of CD4 SP-sorted cells (Table 2, experiments 2, 4, 5 and 6), indicated that the culture conditions utilized in these experiments were not limiting the potential proliferation of the sorted cells in response to any of the stimulants tested. That this response was a recall response is demonstrated by the fact that sorted lymphocytes obtained from a PrV-naive animal did not respond to stimulation with PrV (Table 2, experiment 1). Similarly, prior to immunization, the immune animals (Table 2, experiments 2–8), did not respond to either virus (data not shown). The specificity of the response was demonstrated by the lack of response of any of the cell populations tested to stimulation with either HSV-2 (Table 2, experiments 4 and 5), or SPV (Table 2, experiment 7). That a stimulating dose of SPV was utilized in experiment 7 was indicated by the fact that PBMC from a SPV-immune animal responded strongly to stimulation with the same preparation and dose of SPV (Table 2, experiment 8). Since both CD4 SP and CD4/CD8 DP lymphocytes were able to respond to stimulation with recall viral antigen, these results indicate that, by definition, memory/effector T cells are present within both lymphocyte populations. Sorted CD8 SP lymphocytes were also responsive to recall viral antigen but required the addition of IL-2. The proliferative response of sorted CD8 SP lymphocytes to stimulation with recall viral antigen was very weak (< 2000 c.p.m.), but could be enhanced 10-fold by the addition at the beginning of the culture of 10 U of recombinant human IL-2 (data not shown).

As a control for the manipulation of the lymphocytes (i.e. staining and sorting) the response of stained/unsorted and of untreated cells from the same pool of lymphocytes utilized to isolate the sorted cells is shown. By comparing the response of the untreated cells to the stained/unsorted cells, it is apparent that under the conditions utilized for this study the staining of the cells with anti-CD4 and anti-CD8 antibodies did not appear to have a detectable effect on their ability to proliferate to any of the stimuli tested (Table 2). Because the proportion of CD4$^+$ lymphocytes in the unsorted PBMC populations utilized in these experiments was approximately 30%, the number of unsorted cells cultured per well was set at two and a half times the number of sorted cells cultured per well, i.e. 5×10^5 unsorted cells/well versus 2×10^5 sorted cells/well. Thus, the absolute number of CD4$^+$ cells cultured/well was similar between sorted and unsorted cell populations. In all but one of the sorting experiments (Table 2, experiment 3), the response to PHA was significantly higher in the unsorted cells than in CD4$^+$ sorted cells. A fourfold higher amount of IL-2 is produced in PHA-stimulated lymphocyte cultures as compared to PrV-stimulated cultures (data not shown).

Lymphoblasts in viral antigen-stimulated lymphocyte cultures are predominantly CD4/CD8 DP

To investigate the role of antigen stimulation in the generation of CD4/CD8 DP lymphocytes, we sought to examine the phenotype of lymphoblasts generated in response to PrV and SPV. Proliferative responses to stimulation with recall viral antigen (PrV and SPV) in our blastogenic assay are displayed in Table 2. Similar strong proliferative responses have been consistently induced in swine in our laboratory by immunization with these viruses. The phenotype of lymphoblasts generated as a result of a secondary response to SPV or PrV was determined by two-colour flow cytofluorometry in 5-day cultures of viral antigen-stimulated PBMC from SPV- or PrV-immune pigs, respectively (Fig. 1). Populations of resting (small lymphocytes) and activated (lymphoblasts) cells within a culture were analysed independently for their phenotype based on their characteriztic light scatter (LS) profile. The LS which identifies resting (small) and activated (large) lymphocytes was

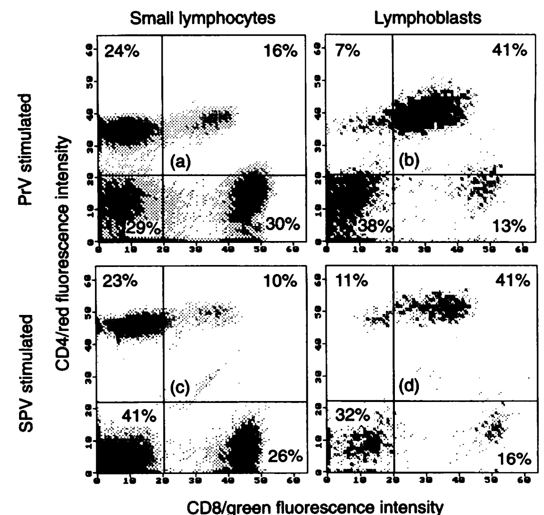

Figure 1. Two-colour cytofluorometric analysis of porcine lymphocytes after stimulation with recall viral antigen. PBMC from a PrV- or SPV-immune swine were stimulated with PrV (a and b) or SPV (c and d), respectively. Cells were harvested after 5 days and stained for CD4 and CD8 expression. Selective cytofluorometric analysis of small lymphocytes (a and c) or lymphoblasts (b and d) was achieved by electronic gating in the dual-parameter display of right angle light scatter versus forward angle light scatter. Numbers represent the percentage of positive cells within each quadrant. Proportions of lymphocyte subsets at the beginning of culture were similar to those expressed by small lymphocytes after 5 days in culture (a and c).

confirmed by morphological and functional analysis of cells which were sorted based on their LS (not shown). Lymphoblasts in virus-stimulated cultures represented on average 10–20% of the total number of viable cells in the culture. No lymphoblasts were detected in unstimulated cultures (not shown). These analyses revealed that the predominant lymphoblast phenotype in both SPV- and PrV-stimulated cultures were CD4/CD8 DP cells which represented 41% of these cells (Fig. 1 b and d). CD4/CD8 double negative (DN) were the second most abundant phenotype accounting for 32–38% of the blasts, followed by CD8 SP and CD4 SP cells which accounted for 13–16% and 7–11% of the lymphoblasts, respectively. The predominance of CD4/CD8 DP lymphoblasts in these cultures was in striking contrast to the proportion of small lymphocytes with this phenotype in the same culture (Fig. 1 a and c). CD4/CD8 DP cells were the least abundant phenotype among small lymphocytes, representing only 10–16% of these cells. Small lymphocytes with CD4/CD8 DN phenotype accounted for 29–42% of the cells, followed by the CD8 SP and CD4 SP lymphocytes which accounted for 26% and 24% of these cells, respectively (Fig. 1 a and c). The proportions of the different lymphocyte subsets detected in the cultured small lymphocytes was very similar to the proportions of the cells before culture. Of note was that the predominance of CD4/CD8 DP lymphoblasts was not seen in lymphocyte cultures stimulated with PHA. These cultures contained mostly CD4 SP and CD8 SP lymphoblasts, and very few, if any, CD4/CD8 DP lymphoblasts (not shown). No double positive cells were detected when control reagents were utilized for the staining. These reagents included isotype matched biotin-labelled or unlabelled mAb specific for irrelevant antigens which were used to replace, respectively, the biotin-labelled anti-CD4 or unlabelled anti-CD8 mAb (not shown). A $F(ab)'_2$ fraction of FITC-labelled rabbit anti-mouse antibody was always used to avoid non-specific FC receptor-mediated binding.

Lymphoblasts derived from viral antigen-stimulated CD4 SP lymphocytes were predominantly CD4/CD8 DP

To examine further the effect of stimulation with recall viral antigen in the generation of CD4/CD8 DP cells, peripheral blood CD4 SP, CD8 SP and CD4/CD8 DP lymphocytes were isolated by FACS from PrV- or SPV-immune pigs. The purity of the sorted cells ranged from 94 to 96% as determined by cytofluorometry immediately after the sort. The phenotype of these cells was again analysed following stimulation for 5 days with PrV or SPV, respectively. Interleukin-2 (10 U/ml) was added to the CD8 SP lymphocyte cultures to enhance their response. As before, the phenotypes of small lymphocytes

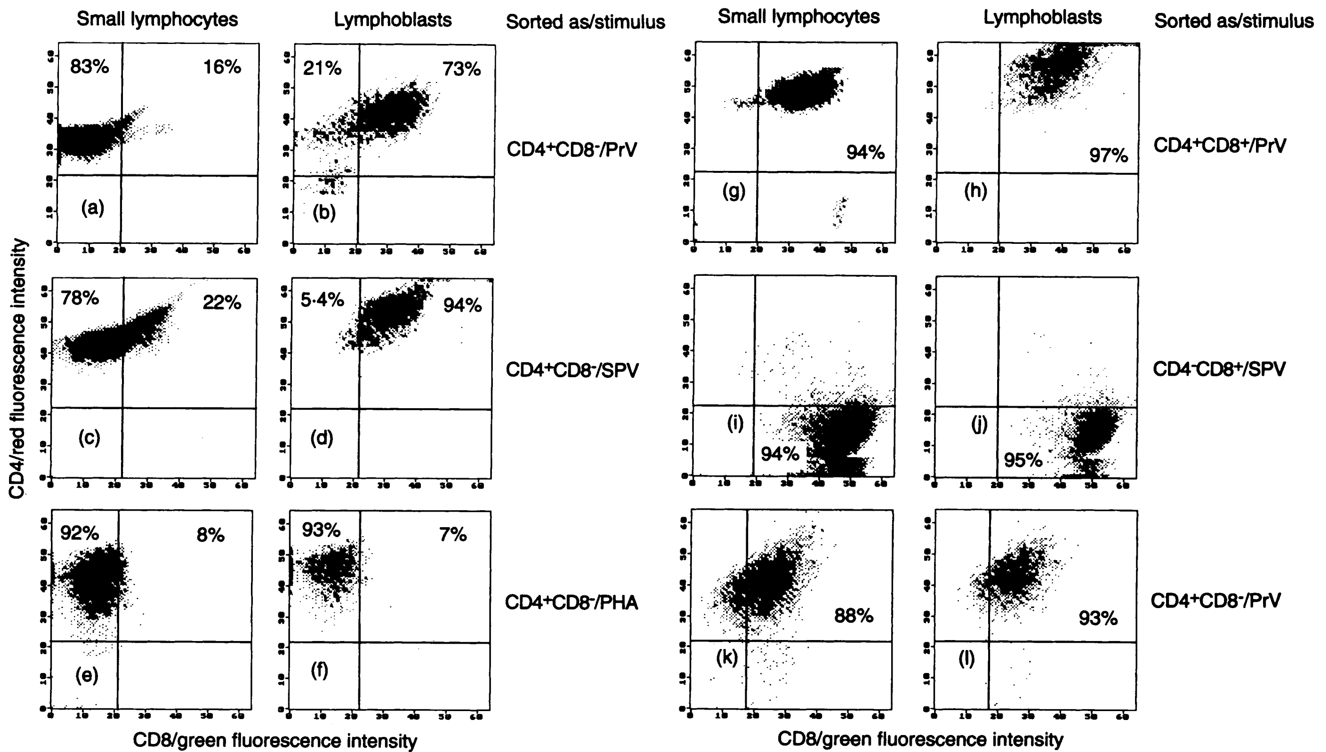

Figure 2. Two-colour cytofluorometric analysis of FACS-purified porcine lymphocytes after stimulation with mitogen or recall viral antigen. Porcine peripheral blood $CD4^+CD8^-$ (a–f), $CD4^+CD8^+$ (g, h) and $CD4^-CD8^+$ (i, j) lymphocytes were isolated by FACS from PrV-immune (a, b, i, j, k, l, e and f) or SPV-immune (c, d) swine and stimulated with PrV (a, b, i, j, k, l), SPV (c and d) or PHA (e and f) in the presence of autologous glass-adherent cells. With the exception of cells in panels k and l, cultured cells were harvested after 5 days in culture and analysed for the expression of CD4 and CD8. Selective cytofluorometric analysis of small lymphocytes (a, c, e, g, i and k) or lymphoblasts (b, d, f, h, j, l) was achieved as described in Fig. 2. Purity of the sorts as determined by analysis of sorted cells before culture was >94% in all experiments. A cell line derived from sorted $CD4^+CD8^-$ lymphocytes (96% pure) was analysed after 5 weeks in culture (k and l).

which did not participate in the response and of lymphoblasts generated in response to stimulation by the viral antigen were analysed separately by gating in the respective cell population based on their light scatter. Pseudorabies virus-induced lymphoblasts derived from CD4/CD8 DP and CD8 SP lymphocytes were of the same phenotype as their progenitor cells (Fig. 2 h and j). In contrast, >90% of lymphoblasts derived from CD4 SP cells in response to stimulation with either PrV or SPV had a CD4/CD8 DP phenotype (Fig. 2 b and d). This result was consistently observed in response to PrV and SPV in four and two independent experiments, respectively. Stimulation of CD4 SP cells with PHA did not stimulate the production of CD4/CD8 DP lymphoblasts (Fig. 2 f).

Sorted CD4 SP lymphocytes with a 96% purity (Table 2, experiment 5), were maintained in culture for 10 weeks by biweekly cycles of stimulation with viral antigen (PrV) plus mitomycin C-treated autologous glass-adherent cells, and by the addition of 10 U/ml of recombinant human IL-2 every 4 days. Samples from these cells were examined for their phenotype at weeks 2, 5 and 10 of culture. The phenotypic analysis of this cell line at the 5th week of culture is shown in Fig. 2, panels (k) and (l). At each one of these time points the cultured cells were found to have a CD4/CD8 DP phenotype, even when some of them had returned to a quiescent state, as determined by their light scatter which was characteristic of small lymphocytes (Fig. 2k) and reduced proliferative activity (data not shown). Together, these experiments suggest that in response to stimulation with recall viral antigen CD4/CD8 DP cells can be generated from sorted CD4 SP cells with an apparently stable phenotype.

Cross-reactivity of anti-human integrin mAb with porcine molecules

To characterize further the CD4/CD8 DP cell population, we typed cell surface molecules which could distinguish between porcine naive and memory/effector cells. Four mAb specific for the human integrin subunits, $\beta1$ (4B4), $\alpha4$ (L25 and 8F2), and $\alpha5$ (2H6), were tested for their cross-reactivity with porcine molecules. Of these, only mAb 4B4 (27) and L25[28] immunoprecipitated proteins from ^{125}I-cell surface labelled porcine peripheral blood leucocytes (Fig. 3). The human $\beta1$ integrin-specific mAb 4B4 immunoprecipitated two well-represented proteins of MW 130 000 and 150 000 (Fig. 3, lane a), and a faint band corresponding to an 80 000 MW protein. Of note was that these three molecules are of similar molecular weight to the molecular complex immunoprecipitated from human leucocytes by antibodies specific for $\beta1$ integrins,[27,31] which is comprised of the intact $\alpha4$ chain (150 000), the $\beta1$ integrin (130 000) and the cleaved form of $\alpha4$ (80 000). Similarly, the human $\alpha4$-specific mAb L25, precipitated two chains with apparent MW of 150 000 and 80 000 (Fig. 3, lane d). In other experiments L25 precipitated a 130 000 protein in addition to the 150 000 and 80 000 proteins (not shown). The molecular complex immunoprecipitated by mAb L25 from porcine PBMC is also similar to the complex recognized by $\alpha4$-specific mAb on human leucocytes.[31] The mAb 8F2 and 2H6 did not appear to react with any porcine leucocyte molecule (Fig. 3, lanes b and c). As a control for the immunoprecipitation, the mAb K252.1E4, specific for porcine CD45[32] is shown. This mAb reacts with at least three related proteins of 190 000,

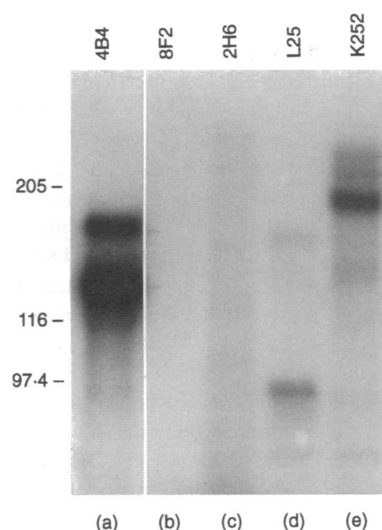

Figure 3. Immunoprecipitation analysis of ^{125}I-labelled porcine leucocytes with mAb specific for molecules of the human $\beta1$ integrin family. Peripheral blood mononuclear cells were cell surface labelled with ^{125}I and immunoprecipitated with mAb 4B4 specific for human $\beta1$ integrin (lane a); mAb 8F2, specific for human $\alpha4$ integrin (lane b); mAb 2H6, specific for human $\alpha5$ integrin (lane c); mAb L25, specific for human $\alpha4$ integrin (lane d); or as a control, with mAb K252·1E4, specific for porcine CD45 (lane e). Precipitates were analysed by 6% polyacrylamide gel electrophoresis under reducing conditions. All lanes represent a 72-hr exposure. The migration of MW markers is indicated.

210 000 and 226 000 (Fig. 3, lane e). Cytofluorometric analyses revealed that swine lymphocytes and monocytes stained with mAb 4B4 displayed distinct staining patterns and mean fluorescence intensities (MFI). Lymphocytes on the one hand displayed a bimodal staining pattern with high fluorescence (MFI = 150) and low fluorescence (MFI = 90) intensities. Monocytes, on the other hand expressed a single peak with a MFI of 170 (not shown). This reactivity pattern seen with porcine lymphocytes and monocytes is typical of the reactivity pattern seen on the respective human cells.[33] Together, these

Table 3. Expression of the 4B4 antigen by peripheral blood T lymphocyte subsets of adult pigs*

% Marker expression on the indicated lymphocyte subset†					
$CD4^+CD8^-$		$CD4^-CD8^+$		$CD4^+CD8^+$	
$4B4^{hi}$	$4B4^{lo}$	$4B4^{hi}$	$4B4^{lo}$	$4B4^{hi}$	$4B4^{lo}$
20·4 ± 8	75·5 ± 7	45·0 ± 8	52·1 ± 9	74·6 ± 7	23·6 ± 7

*List mode files of PBMC samples analysed by three-colour cytofluorometry for: CD4, CD8, and 4B4 antigen expression, were electronically gated on $CD4^+CD8^-$, $CD4^+CD8^+$, and $CD4^-CD8^+$ lymphocytes and the proportion of $4B4^{hi}$ and $4B4^{lo}$ cells was determined. Three-colour staining was done as described in the Materials and Methods. 4B4 staining defined as low was from channel 50 to 125, and that greater than channel 125 was designated as high.

†Percentages represent the mean ± SD of 12 adult pigs, ranging in age from 12 to 20 months.

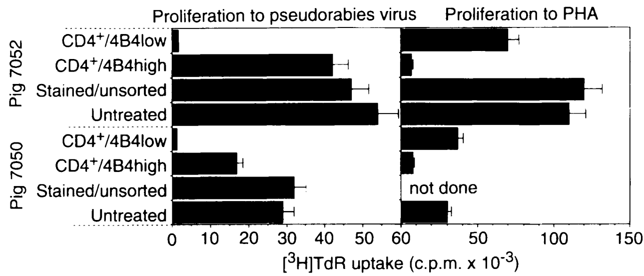

Figure 4. Proliferative response of $CD4^+CD29^{hi}$ and $CD4^-CD29^{lo}$ lymphocytes to stimulation with mitogen or recall viral antigen. Peripheral blood mononuclear cells from PrV-immune swine were stained for CD4 and CD29 expression as described in the Materials and Methods. Cells were separated by FACS into $CD4^+CD29^{hi}$ and $CD4^+CD29^{lo}$ subsets. Sorted cells were stimulated with either PrV (left panel) or PHA (right panel). Stained but unsorted and untreated cells from the same pool of lymphocytes utilized for each experiment were also tested as controls of the effect of the experimental manipulations. Values for proliferation represent mean cpm of triplicate cultures. Background proliferation was less than 500 c.p.m. in all samples.

data suggest that mAb 4B4 cross-reacts with the porcine homologue of the human $\beta1$ integrin molecule also known as CD29.

CD4/CD8 DP lymphocytes predominantly express high levels of the 4B4 antigen

Normal human peripheral blood lymphocytes have been subdivided into subpopulations expressing high and low levels of the integrin $\beta1$ subunit, as assessed using the mAb 4B4.[27] Since we observed that staining with this mAb separated porcine lymphocytes in a similar pattern, we examined the differential expression of the 4B4 antigen on porcine T-cell subsets by three-colour cytofluorometry. This analysis revealed that porcine CD4 SP, CD8 SP and CD4/CD8 DP lymphocytes could each be divided, according to their level of the 4B4 antigen expression, into subsets expressing high levels ($4B4^{hi}$) and low levels ($4B4^{lo}$) of this cell surface antigen. The mean percentage of $4B4^{hi}$ and $4B4^{lo}$ within each subset was calculated from the analyses of PBMC obtained from 12 pigs between 1–2 years of age (Table 3). On average, 75% of the CD4 SP cells were $4B4^{lo}$, while 74% of the CD4/CD8 DP lymphocytes were $4B4^{hi}$. In contrast, in the CD8 SP lymphocyte population there was no consistent predominance of either $4B4^{hi}$ or $4B4^{lo}$ cells.

Enhanced proliferation of the $CD4^+4B4^{hi}$ lymphocyte subset to recall viral antigen

To analyse the functional properties of $CD4^+$ cells with high- and low-level expression of the 4B4 antigen, $CD4^+$ cells isolated from PrV-immune swine were separated by FACS into $CD4^+4B4^{hi}$ and $CD4^+4B4.^{lo}$ Cytofluorometric analysis of sorted cells showed that the purity of the sorted cells was >95% for both cell populations. The two subpopulations exhibited responses that were reciprocal to the stimulation with lectin or recall viral antigen. Stimulation with PrV resulted in strong proliferation of the $CD4^+4B4^{hi}$ subset, while the $CD4^+4B4^{lo}$ subset showed only a minimal response (Fig. 4, left panel). In contrast, the $CD4^+4B4^{lo}$ subset proliferated with three- to five-fold greater intensity to PHA than the

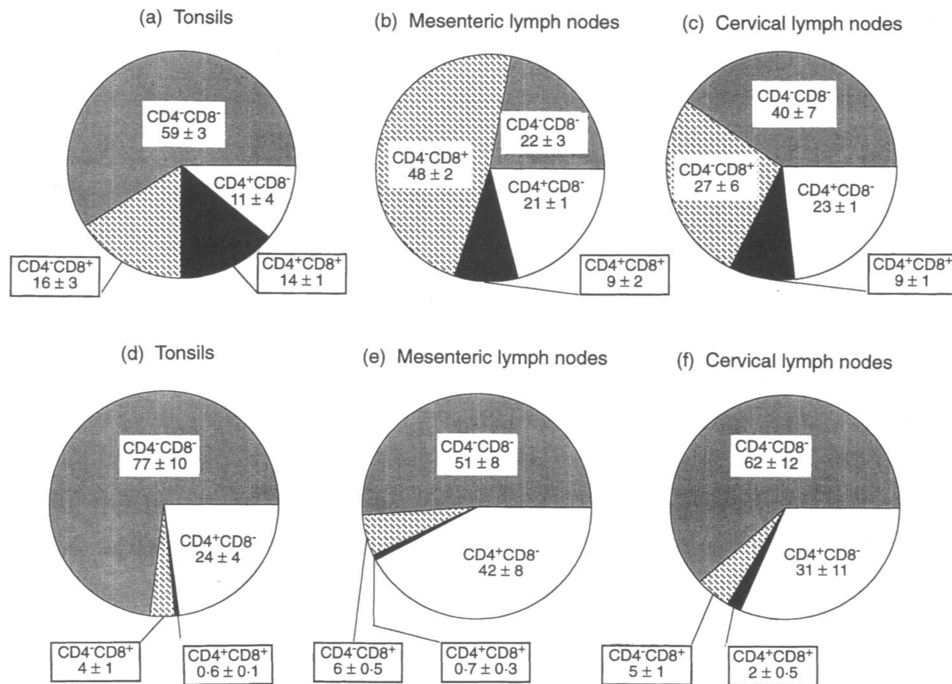

Figure 5. Two-colour cytofluorometric analysis of CD4 and CD8 expression by lymphocytes from tonsil and mesenteric and cervical lymph nodes of swine. Single cell suspensions of the indicated tissues were prepared from swine at 6 months (panels a, b and c) or 3 days of age (panels d, e and f), stained and analysed for CD4 and CD8 expression as described in the Materials and Methods. Numbers represent the mean percentage ± SEM from the analysis of cells from three pigs.

CD4$^+$4B4^{hi} subset (Fig. 4, right panel). The proportion of CD4/CD8 DP and of CD4 SP cells in the PBMC of the pigs utilized in these experiments was 10% and 20%, respectively.

Distribution of CD4$^+$CD8$^+$ T cells in secondary lymphoid tissues

Cell suspensions obtained from palatine tonsils and mesenteric and cervical lymph nodes, sampled from 6–7-month-old pigs, were subjected to two-colour cytofluorometric analysis for the expression of CD4 and CD8. In each of three animals, the proportion of CD4/CD8 DP cells in the palatine tonsils accounted for more than 50% of the total CD4$^+$ lymphocyte population (Fig. 5a). In contrast, in the mesenteric and cervical lymph nodes of the same animals, CD4/CD8 DP cells represented only one-third or less of the total percentage of CD4$^+$ cells, with the majority of these cells being CD4 SP (Fig. 5b and c). The relative abundance of DP cells in the tonsils of young adult animals was in striking contrast to the complete lack of CD4/CD8 DP lymphocytes in the tonsils and lymph nodes of 3-day-old swine in which all CD4$^+$ cells were CD4 SP cells (Fig. 5 d, e and f).

DISCUSSION

We have focused on the biology of porcine CD4/CD8 DP lymphocytes. These cells were known to be abundant (reportedly up to 60% of total lymphocytes) in the peripheral blood of adult swine.[3] However, the significance of this population or its temporal appearance in the life of pigs was unknown.[4] The longitudinal and cross-sectional studies, involving a total of 103 pigs, clearly establish that the appearance of this lymphocyte population is strongly associated with age ($R^2 = 0{\cdot}9$) in outbred pigs as well as partially inbred NIH miniature pigs. The data showed both a qualitative and quantitative change in the peripheral blood of aging pigs marked by an increase in the percentage of CD4/CD8 DP cells and a significant increase in their absolute number in the circulation (Table 1). The similarity in the percentages of CD4/CD8 DP lymphocytes between the outbred group of pigs and the younger group of partially inbred NIH miniature pigs at different ages indicates that the rate of accumulation of CD4/CD8 DP cells with age, as well as the proportions of this lymphocyte subset at different ages in these two groups is the same. An apparent exception were the two older NIH miniature pigs which were first sampled at 27 months of age. These two animals seemed to be different in that they had a higher percentage (30%) of CD4/CD8 DP cells at 27 months of age as compared to the 19% present in the younger group of NIH swine at 26 months of age. However, because of a lower lymphocyte count in the two older pigs, these differences were not evident when the absolute number of CD4/CD8 DP lymphocyte per ml of blood was calculated (Table 1). The two older miniature swine also had a high proportion of CD4/CD8 DP at 46 and 52 months of age. However, the percentage of CD4/CD8 DP lymphocytes in the oldest oubred pigs examined (45–54 months old) was also high, and ranged from 29% to 42%. Thus, the relatively high percentage of CD4/CD8 DP cells in these two older miniature swine is not that uncommon for pigs at this age. The high percentage of CD4/CD8 DP cells in the peripheral blood of pigs (30–55% of total lymphocytes) detected in this study agrees with the high range of values previously reported for this lymphocyte subset.[3]

From the sorting experiments we established that cells in the CD4/CD8 DP lymphocyte subset are responsive to stimulation with optimal doses of recall viral antigen (10 μg/ml), indicating that memory/effector cells are present in this cell population (Table 2). The demonstration that the CD4$^+$/4B4^{hi} cells also responded to stimulation with recall viral antigen, while CD4$^+$/4B4^{lo} did not, is evidence that the increased expression of this antigen is a reflection of the functional differentiation of porcine CD4$^+$ lymphocytes into memory/effector cells. Therefore, the level of 4B4 antigen expression can be used to distinguish between CD4$^+$ naive and memory/effector cells. The observation that the CD4/CD8 DP lymphocyte subset is predominantly (75%) 4B4^{hi}, suggests that this lymphocyte subset is comprised mostly of memory T cells, while the CD4 SP lymphocyte subset, which is predominantly 4B4^{lo} (75%), is mostly comprised of naive cells. It was interesting therefore that even though 4B4^{hi} cells were three times more abundant among CD4/CD8 DP cells than among CD4 SP (Table 3), both cell populations responded with equal intensity to stimulation with recall viral antigen (Fig. 4). This result could be due to differences in the ability of these T-cell subsets to secrete IL-2 in response to recall viral antigen, this being manifested by a strong proliferative response. This possibility remains to be examined. The CD4 SP cells (Table 2) and CD4$^+$/4B4^{lo} lymphocytes (Fig. 4), were several times more responsive to PHA than CD4/CD8 DP or CD4$^+$/4B4^{hi} cells. Human CD4$^+$ memory cells are 4B4^{hi}, respond strongly to recall antigen and poorly to mitogen, while naive CD4$^+$ cells are 4B4^{lo}, proliferate strongly in response to stimulation with mitogens such as PHA but not to recall antigen.[27,34] Thus, the data shown here for pigs are in agreement with the phenotype and responsiveness of human naive and memory/effector cells to recall antigen and mitogen.

The differential expression of certain cell surface molecules has been used to distinguish between naive and memory/effector cells. Human memory/effector T lymphocytes have been shown to express increased levels of several adhesion molecules including lymphocyte function-associated antigen-3 (LFA-3), LFA-1, CD44, CD2 and the $\beta 1$ integrin.[35] Of these, the integrins are a family of highly conserved molecules across species with a >90% amino acid identity between human and mouse $\beta 1$ integrins.[36] This remarkable similarity is reflected by cross-reactivity of antibodies directed against this molecule between vertebrates, invertebrates and fungi.[37] The mAb 4B4, which recognizes the human $\beta 1$ integrin,[27] was found to cross-react with porcine leucocytes, generating very similar staining patterns with porcine lymphocytes and monocytes as the staining patterns seen with the respective human cells stained with antibodies specific for human $\beta 1$ integrin.[33] Immunoprecipitation of ^{125}I-cell surface labelled porcine PBMC with mAb 4B4 yielded three molecules represented by two strong bands of 130 000 and 150 000 MW and a faint band of 80 000 MW. The size of these molecules is very similar to that of the molecular complex immunoprecipitated by $\beta 1$ integrin-specific mAb from human leucocytes, which is comprised of the intact $\alpha 4$ integrin (150 000), the $\beta 1$ (130 000) integrin, and the cleaved form of the $\alpha 4$ integrin (80 000 and 70 000).[31,33] Given the high degree of conservation across species for the $\beta 1$ integrin,[36] the cross-reactivity detected in the present study is not surprising. These

results suggest, that the human β1 integrin-specific mAb 4B4 recognizes the porcine homologue of human CD29. The most important observation is, however, that mAb 4B4 reacts with a molecule which expressed at high and low levels on the cell surface of porcine T cells, and generates a bimodal staining pattern which can be used to distinguish functionally between naive and memory/effector porcine T cells.

Although at the beginning of the culture of unfractionated resting lymphocytes, CD4/CD8 DP lymphocytes were the least abundant subset, after 5 days, CD4/CD8 DP lymphoblasts represented the predominant activated cell phenotype in cultures stimulated with recall viral antigen. In contrast, CD4 SP lymphoblasts were almost non-existent, even though potential progenitors for these cells were well represented among the starting lymphocytes in culture. Similar observations have been made in porcine lymphocyte cultures stimulated with alloantigen[3] or helminth antigen.[22] Notably, recall viral antigen-induced lymphoblasts derived from electronically sorted CD4 SP cells after 5 days in culture were also 73–94% CD4/CD8 DP, while lymphoblasts derived from CD4/CD8 DP and CD8 SP cells maintained their DP and SP phenotype, respectively. Several controls were performed to eliminate the possibility of artefactual double-positive staining of lymphoblasts. The best control, however, is the fact that stimulation of CD4 SP T cells with PHA did not result in the generation of CD4/CD8 DP blasts (Fig. 2f), arguing strongly that the CD4/CD8 DP lymphoblasts detected in the virus-stimulated cultures were not the result of a staining artefact. These results indicate a role for recall antigen in the induction of cells with CD4/CD8 DP phenotype, and also suggest that CD4 SP cells might have switched their phenotype to a CD4/CD8 DP cell (Fig. 2 b and d). However, because the purity of the sorted cells at the beginning of the culture was 94–96% we cannot rule out the possibility that a small proportion of contaminating CD4/CD8 DP cells might have given rise to CD4/CD8 DP lymphoblasts. The fact that both CD4 SP and CD4/CD8 DP cells were capable of proliferating with similar intensity to recall viral antigen would suggest that at least some of these DP lymphoblasts could have been derived from single positive cells. Regardless of their origin, it is clear from this study that stimulation with specific antigen plays a role in the generation of CD4/CD8 DP cells *in vitro*.

While in the palatine tonsils of newborn swine CD4/CD8 DP lymphocytes were almost non-existent, in the tonsils of 6–7-month-old pigs this lymphocyte susbset was in equal or larger proportion to CD4 SP cells. In contrast, CD4 SP cells were the predominant $CD4^+$ phenotype in the lymph nodes of the 6–7-month-old pigs, accounting for two-thirds of these cells. These observations raise the possibility that the tonsil is a site where the CD4/CD8 DP lymphocyte subset is generated. Tonsils are immunologically active lymphoid organs[37] from which pathogens can frequently be isolated.[39,40] Thus, activated lymphocytes and cells with a memory/effector phenotype would be expected in high proportions in this organ. Accordingly, T cells spontaneously secreting cytokines, and cells with a memory/effector phenotype are abundant in human tonsils.[38,41] Additional evidence that CD4/CD8 DP cells are present in sites where intense antigenic stimulation occurs, are the reports that this lymphocyte subset can be found in the joint fluid of patients with juvenile rheumatoid arthritis,[9] among the lymphocytes infiltrating human and porcine renal allografts,[10,11] and also in significant percentages (> 15% of total peripheral blood lymphocytes) in the peripheral blood of patients suffering from neoplasia.[8] Furthermore, the appearance of CD4/CD8 DP iIEL in rats is dependent on both age and the presence of the gastrointestinal flora.[14] Together, available evidence argues in favour of the possibility of a stimulatory influence of environmental antigens on the appearance of CD4/CD8 DP cells. The evidence shown here that antigen stimulation results in the generation of double-positive cells strongly supports this postulate.

Evidence was presented which supports the hypothesis that porcine CD4/CD8 DP cell population includes memory cells. The presence of this distinct population in the immune system of healthy aged swine is likely to be related to a helper, immunoregulatory and/or immunosurveillance function. CD4/CD8 DP cells are known to express class II MHC antigens,[42] and thus, can potentially present antigen to other cells. Because of the proposed use of pig organs for xenotransplantation into humans, the likely possibility that these cells may be transferred along with these organs as passenger lymphocytes needs to be considered. This possibility, along with the observations that lymphocytes with CD4/CD8 DP phenotype can be found in certain pathological states in humans, highlights the importance of understanding the biological significance of this lymphocyte population and their possible use in immunomodulating strategies.

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