Immunology 1996 **88** 238–246

Differentiation between MHC-restricted and non-MHC-restricted porcine cytolytic T lymphocytes

T. PAULY,* E. WEILAND,* W. HIRT,† C. DREYER-BUX,* S. MAURER,* A. SUMMERFIELD‡ & A. SAALMÜLLER* **Federal Research Centre for Virus Diseases of Animals, Tübingen,* †*Orpegen, Medizinisch-Molekularbiologische, Forschungsgesellschaft m. b. H., Heidelberg, Germany,* and ‡*Institut für Viruskrankheiten und Immunprophylaxe, Switzerland*

SUMMARY

The immune system of swine is unique in that the expression of CD4 and CD8 antigens defines four subpopulations of resting extrathymic T lymphocytes. Beyond phenotypic differences to other species, porcine T lymphocytes, particularly when derived from infected animals, are known to show high non-specific cytolytic *in vitro* activity. Here we describe the putative porcine CD6 antigen (workshop CD6; wCD6) which enables a phenotypic separation of T lymphocytes responsible for major histocompatibility complex (MHC)-restricted and non-MHC-restricted cytotoxicity. The putative porcine CD6 analogue, wCD6, a protein with a molecular mass of 110 000, shows high specificity for T lymphocytes and is neither expressed on B lymphocytes nor on cells of the myeloid lineage. In the extrathymic T-lymphocyte compartment wCD6 characterizes two T-lymphocyte fractions: $wCD6^+$ T lymphocytes including both $CD4^+$ T-helper cell subpopulations ($CD4^+CD8^-$ and $CD4^+CD8^+$) and within the $CD4^-CD8^+$ fraction cells with high CD8 antigen density. In contrast the $CD4^-CD8^-$ γ/δ T-cell receptor (TCR) subset and $CD4^-CD8^+$ cells with low CD8 antigen density are included in the $wCD6^-$ T-lymphocyte fraction. Functional studies with separated wCD6 fractions revealed that the $wCD6^-$ cells can be characterized by spontaneous and non-MHC restricted cytolytic activity, whereas the $wCD6^+$ T lymphocytes are responsible for MHC-restricted T-cell functions. Thus, the porcine wCD6 is an important antigen to discriminate between MHC-restricted and non-MHC-restricted cytotoxicity.

INTRODUCTION

Previous work has revealed striking differences in the peripheral T-lymphocyte compartments of swine compared with other species. The most important difference is the existence of four extrathymic T-lymphocyte subpopulations defined by the expression of the differentiation antigens CD4 and CD8.[1–3] Besides the classical phenotypes of $CD4^+CD8^-$ T-helper cells and $CD8^+CD4^-$ cytolytic T-lymphocytes, $CD4^-CD8^-$ and $CD4^+CD8^+$ T-cell subpopulations exist in peripheral blood as well as in lymphoid tissues. While T-helper function can be attributed to $CD4^+$ cells, either positive or negative for CD8,[4] the specific function of the $CD4^-CD8^-$ subpopulation is still unclear. Preliminary results indicate non-specific functions of this heterogeneous subpopulation containing a high amount of $\gamma\delta$ T-cell receptor (TCR) positive cells.[5–8] Recently, the $CD4^-CD8^+$ cytolytic T-lymphocyte subpopulation could be discriminated by the expression of CD5 into a $CD5^-$ and a $CD5^+$ fraction. Whereas the $CD4^-CD5^-CD8^+$ fraction contains cells with spontaneous cytolytic activity, the $CD4^-CD5^+CD8^+$ T lymphocyte subset includes the progenitors of the major histocompatibility complex (MHC)-restricted cytolytic T lymphocytes.[9] In this report we focus on the expression of the putative porcine CD6 antigen characterized by the monoclonal antibody (mAb) a38b2[10,11] and the *in vitro* function of the CD6-defined T-lymphocyte fractions. This antigen is assigned according to the rules for the nomenclature of Swine CD accepted in the 'Second International Swine CD workshop', because of the lack of any sequence data as workshop CD6 or wCD6.[12]

Received 30 November 1995; revised 24 February 1996; accepted 25 February 1996.

Correspondence: Dr Armin Saalmüller, Federal Research Centre for Virus Diseases of Animals, PO Box 1149, 72001 Tübingen, Germany.

MATERIAL AND METHODS

Cells

Throughout the study 6-month-old swine of German Landrace served as cell donors. Thymocytes and cells from mesenteric lymph nodes were prepared in cell suspensions. Peripheral blood mononuclear cells (PBMC) and lymph node mononuclear cells were enriched by Ficoll–Hypaque (Pharmacia, Uppsala, Sweden) centrifugation. For purification of T lymphocytes, monocytes were depleted by plastic-adherence[13]

© 1996 Blackwell Science Ltd

and B lymphocytes by passages over nylon-wool columns.[2] K562 tumour cells were used as target cells for the quantification of the spontaneous cytolytic activity.[14]

Monoclonal antibodies

Anti-CD2 (mAb MSA4; mouse IgG2a[10,15]), anti-CD4 (mAb 74-12-4; mouse IgG2b[10,13]) and anti-SWC3 (mAb 74-22-15A; mouse IgG2b[12,16]) were received from Dr J. K. Lunney (USDA, ARS, Beltsville, MD). Anti-CD8 (mAb 11/295/33; mouse IgG2a[10,17]), anti-CD5 (mAb b53b7; mouse IgG1[9,10]) and anti-CD6 (mAb a38b2; mouse IgG1[10]) were established in our laboratory.

Immunoprecipitation

Radioimmunoprecipitations were performed as described,[9,18] using the lactoperoxidase technique for labelling of T lymphocytes with Na^{125}.

Multi-colour flow cytometric (FCM) analysis

Labelling of PBMC for the determination of the reactivity of a38b2 with B lymphocytes and monocytes was achieved in a three-step procedure: incubation with rabbit immunoglobulin (Jackson Laboratories, Avondale, PA); labelling with mAb a38b2 (anti-CD6, IgG1), fluorescein isothiocyanate (FITC)-conjugated 74-22-15 (anti-SWC3, IgG2b), and biotinylated anti porcine-immunoglobulin antiserum (Jackson Laboratories); and staining with phycoerythrin (PE)-conjugated anti-mouse IgG1 specific antiserum (Southern Biotechnology, Birmingham, AL) and Cy-5-conjugated streptavidin (Jackson Laboratories, Avondale, PA).

Labelling of thymocytes and purified T lymphocytes derived from blood and mesenteric lymph nodes was performed in a two-step procedure: incubation with mAb a38b2 (anti-CD6, IgG1) or mAb b53b7 (anti-CD5, IgG1) together with mAb MSA4 (anti-CD2, IgG2a) followed by staining with FITC-conjugated anti-mouse IgG2a and PE-conjugated anti-mouse IgG1 antisera (Southern Biotechnology).

Labelling of thymocytes and purified T lymphocytes for three-colour FCM was accomplished by a two-step procedure: incubation with mAb 74-12-4 (anti-CD4, IgG2b), mAb a38b2 (anti-CD6, IgG1) and biotinylated mAb 11/295/33 (anti-CD8, IgG2a); and staining with isotype-specific antisera (PE-conjugated anti-IgG2a and FITC-conjugated anti-IgG2b, respectively; Southern Biotechnology) together with Cy-5-conjugated streptavidin (Jackson Laboratories). All measurements were performed on a dual-LASER FACStar plus (Becton Dickinson, Mountain View, CA) as described previously [18].

The list mode data were processed for the respective figures using PC-lysis™ and Corel draw™ software.

Immunocytochemistry

Cryostat sections (5–8 μm) of porcine mesenteric lymph nodes, tonsils and spleen on poly-D-lysine-coated slides were fixed with 4% paraformaldehyde containing 0·1 M sodium phosphate, 5 mM $MgCl_2$ (10 min, room temperature) and washed three times (5 min) with phosphate-buffered saline (PBS). Endogenous peroxidase was blocked by incubation for 30 min with 0·3% (v/v) H_2O_2 in methanol. After three washing steps with PBS (5 min), non-specific binding of the first mAb was blocked by incubation with PBS containing 1% bovine serum albumin (BSA), 5% horse serum, 5% goat serum, 0·1% Triton-X-100 and 0·1% Tween-20 for 30 min. Labelling with the first mAb (containing 2% milk powder) was performed overnight at 4°. After washing with PBS (3 × 5 min), the slides were stained with biotinylated $F(ab')_2$ fragments of a goat anti-mouse antiserum directed against $F(ab')_2$ fragments (Jackson Laboratories). After five washing steps with PBS, the third one with PBS containing 0·1% (v/v) Triton-X-100, slides were labelled for 30 min using Vectastain Elite ABC KIT (Vector, Burlingham, AL) diluted with PBS containing 2% (w/v) milk powder. After washing (3 × 5 min PBS) antibody binding was visualized by adding DAB (diaminobenzidinetetrahydrochloride, 1 mg/ml in 0·1 M Tris–HCl, 0·01% H_2O_2, pH 7·4) for 5–30 min. The reaction was stopped with H_2O for 5 min. The slides were then mounted with glycerol gelatine solution and analysed in the microscope.

Cell sorting

Separation of CD6-defined T-lymphocyte fractions was achieved by immunomagnetic cell sorting. For this, T lymphocytes were labelled in a 4-step procedure with mAb a38b2 (anti-CD6); biotinylated anti-mouse immunoglobulin (Jackson Laboratories); FITC-conjugated streptavidin (Jackson Laboratories); and biotinylated magnetic microparticles (Miltenyi Biotec, Bergisch Gladbach, Germany). Cell-sorting was performed with a magnetic cell sorter (MACS, Miltenyi Biotec) as described.[19] The purity of all separated fractions was higher than 98% as assessed by FCM.

Cytolytic assays

Cytolytic assays were performed as described.[9,20] Determination of classical swine fever (CSF) virus-specific cytolytic activity of T lymphocytes has been described recently.[21]

RESULTS

Specificity and molecular characterization of the antigen recognized by mAb a38b2

Flow cytometric analyses of the expression of the antigen recognized by mAb a38b2 on porcine PBMC in combination with antibodies against B-lymphocytes and myeloid cells strongly suggested that mAb a38b2 detected an antigen which is exclusively expressed on T lymphocytes (Fig. 1b, c). Neither monocytes expressing the porcine SWC3 antigen (Fig. 1b, quadrant IV, 7% for this individual) nor B lymphocytes characterized by surface immunoglobulin molecules (Fig. 1c; quadrant IV, 24%) showed expression of the antigen recognized by mAb a38b2 (Fig. 1b, c; quadrant I, 34%).

Radioimmunoprecipitation studies identified the a38b2 antigen on ^{125}I surface labelled T lymphocytes (Fig. 1a) as a monomeric molecule with an apparent molecular weight of 100 000–120 000 under reducing (lane 2) and non-reducing (lane 4) conditions. Comparison of FCM and biochemical data with the surface expression and known molecular masses of differentiation antigens from other species strongly suggest that mAb a38b2 recognizes the putative porcine CD6 analogue, whereas the final proof, the sequence of the antigen is still missing. In accord with the rules for the porcine CD nomenclature accepted in the 'Second International Swine CD workshop' this antigen is assigned as wCD6 (workshop CD6).[12]

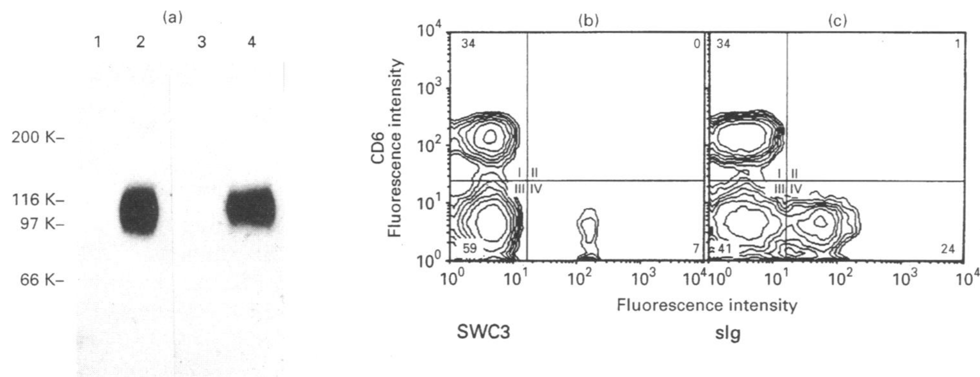

Figure 1. Molecular characterization of the antigen recognized by mAb a38b2 and its surface expression on PBMC. T lymphocytes were labelled with Na^{125} using the lactoperoxidase technique. The molecular mass of the molecule precipitated by mAb a38b2 was determined under reducing (a; lane 2) and non reducing (lane 4) conditions. Lanes 1 and 3 served as controls using an irrelevant mAb for the precipitation. PBMC were labelled with mAb a38b2 together with a mAb against the monocyte/granulocyte-specific SWC3 antigen (b, mAb 74-22-15) or with a polyclonal anti-swine immunoglobulin antiserum (c, sIg). The fluorescence intensities of the respective analyses are displayed as a two-dimensional contour plots with contour lines representing levels of 20, 40, 60, 80, 100, 120 and 140 cells with a total number of 25 000 cells analysed. Quadrants I–IV are defined by background staining with fluorescent conjugates alone. Numbers in the corners indicate the percentages of cells in the respective quadrants.

Coexpression of wCD6 with the T cell-specific CD2 antigen

The data shown in Fig. 1 suggested T cell specificity of the wCD6 antigen, however only a subset of the T-lymphocyte fraction appears to express the wCD6 molecule. To characterize the CD6 positive T-lymphocyte subsets, thymocytes and nylon-wool purified T lymphocytes derived from blood (peripheral blood T lymphocytes, PBTL) and mesenteric lymph nodes (lymph node T lymphocytes, LNTL) were analysed for their CD6 expression in two-colour FCM in combination with mAb against CD2 (Fig. 2a–c).[15] In order to compare the CD6 expression on CD2-defined cell fractions with the CD5 expression,. CD2/CD5-labelled probes were analysed in the same experiment (Fig. 2d–f).

The majority of porcine thymocytes were positive for CD2 (Fig. 2a and d, 96 or 97% for both quadrants II and IV). In the CD2-positive thymocyte fraction a homogeneous weak CD6 expression was found (Fig. 2a). Very few thymocytes with the phenotype $CD2^-CD6^+$ could be detected. CD5 showed an expression pattern similar to CD6, but the antigen density on the $CD2^+$ thymocytes seemed to be higher. A restricted number of $CD2^+$ thymocytes reproducibly exhibited an elevated CD5 antigen density which is seen by a characteristic 'CD5-tailing' (Fig. 2d), which may represent thymocytes with the phenotype of mature T lymphocytes.[9]

PBTL were divided by their CD2 expression into a $CD2^+$ and a $CD2^-$ subset (Fig. 2b, e). Whereas the $CD2^-$ fraction (quadrants I and III), formerly described as null cells[22] and containing the majority of porcine TCR $\gamma\delta$ T lymphocytes,[23] was negative for CD6 (Fig. 2b, quadrant III, 23%), $CD2^+$ T lymphocytes could be separated by their CD6 expression into two a $CD6^+$ (quadrant II, 47%) and $CD6^-$ (quadrant IV, 29%) cell fraction. Regarding the CD5 antigen expression, an identical staining pattern could be demonstrated for the $CD2^+$ T-lymphocyte fraction, which was divided into a $CD2^+CD5^+$ (quadrant II, 47%) and a $CD2^+CD5^-$ (IV, 30%) subset. Compared to the CD6-staining pattern the $CD2^-$ null cells displayed low CD5 expression (Fig. 2e, quadrant I, 20%), but were $wCD6^-$ (Fig. 2b, quadrant III, 23%). Thus, the phenotype of porcine null cells could be defined as $CD5^{+low}$ $CD6^-$ (Fig. 2c, f).

Analyses of T lymphocytes derived from mesenteric lymph nodes (LNTL) revealed a heterogeneous CD2, CD6 and CD5 antigen expression pattern. Only distinct fractions of $CD2^+CD6^+$ and $CD2^+CD5^+$ LNTL existed (Fig. 2c, f, quadrant II, 66% and 77%, respectively). Besides these populations, a mixture of cells with heterogeneous antigen density was detectable, which could not be distributed to uniform subpopulations.

Tissue distribution of $wCD6^+$ T lymphocytes

Because of the peculiar and unusual anatomy of the porcine lymphoreticular tissue[24] it was interesting to determine the distribution of $CD6^+$ T lymphocytes in lymphoid organs in comparison to the distribution of $CD2^+$ and $CD5^+$ T lymphocytes.

In mesenteric lymph node a great number of cells of the perifollicular and interfollicular T-cell areas showed CD6 expression (Fig. 3a). B-cell areas like lymph node follicles contained only few $CD6^+$ cells. A similar distribution was found for CD2 (Fig. 3d) and CD5 (Fig. 3g) positive cells, with the exception of a high number of $CD2^+$ cells in the follicular area (Fig. 3d). All three mAb enabled a clear separation between T- and B-cell areas in mesenteric lymph nodes. The CD6-staining pattern of tonsils was compatible to that of lymph node sections (Fig. 3b). Cells in the interfollicular areas were intensively stained by anti-CD6 mAb, whereas the follicles usually contained only a few CD6-positive cells. Labelling with mAb against CD2 (Fig. 3e) and CD5 (Fig. 3h) showed a similar distribution, but the percentage of cells expressing both antigens seemed to be enhanced in the follicular B-cell areas compared with the lymph node follicles. In spleen CD2 (Fig. 3f), CD5 (Fig. 3i), and CD6 (Fig. 3c) positive cells were mainly found in the T-cell areas of the periarteriolar

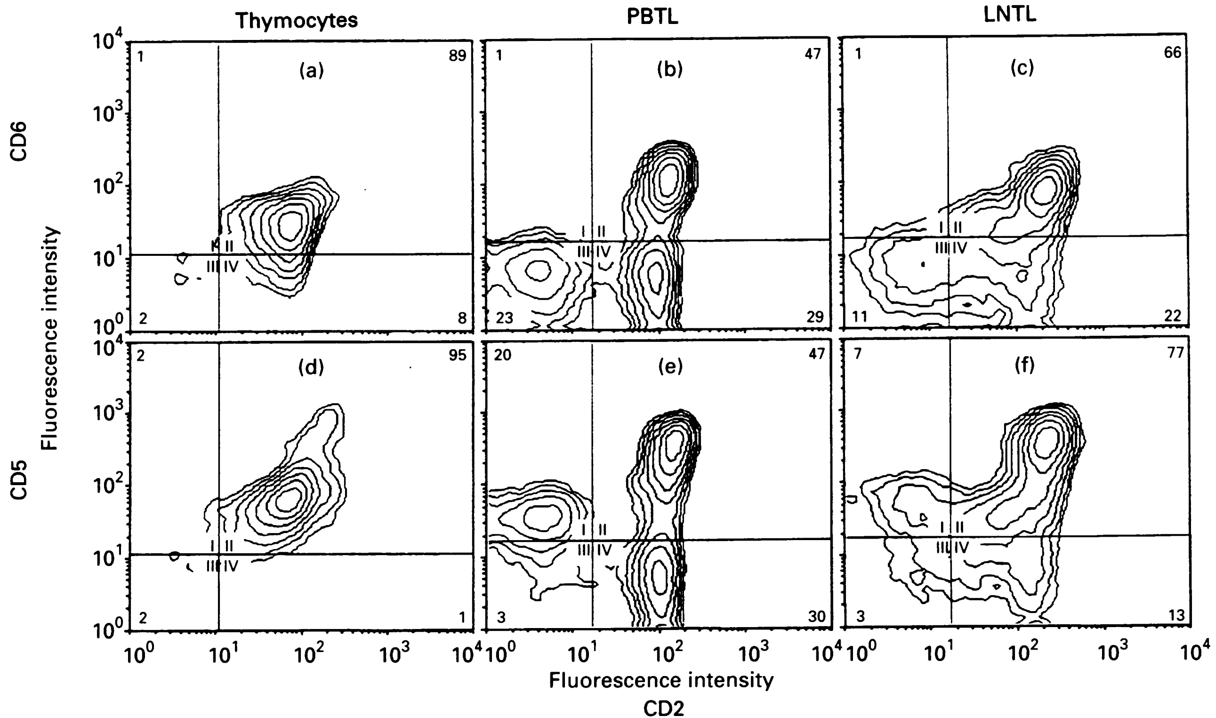

Figure 2. Expression of wCD6 on cells derived from lymphoid tissue. Two-colour FCM analyses of porcine thymocytes, peripheral blood T lymphocytes (PBTL) and T lymphocytes derived from mesenteric lymph nodes (LNTL) labelled with mAb against CD2, CD5 and CD6. The fluorescence intensities of CD2-FITC versus CD6-PE (a–c) and CD2-FITC versus CD5-PE (d–f) are displayed as two-dimensional contour plots with contour lines as described in Fig. 1. Quadrants I–IV are defined by background staining with fluorescent conjugates alone. Numbers in the corners indicate the percentages of cells in the respective quadrants.

lymphoid sheath (PALS), which is represented by cells surrounding the branches of the central splenic arterioles.

Expression of wCD6 on CD4/CD8-defined thymic subpopulations

The expression of the putative CD6 on porcine thymocytes could be characterized as homogeneous and weak, with one peak defining an antigen density which included cells with negative up to intermediate fluorescence intensity (Fig. 2a). This CD6 expression might be dependent of the differentiation stage of the respective thymocytes analysed. To correlate thymic differentiation stages with their CD6 expression, thymocytes were analysed for their CD6 antigen density in three-colour FCM in combination with mAb directed against CD4 and CD8. In analogy to other species[25,26] porcine thymocytes could be divided by their CD4/CD8 expression into four subpopulations (Fig. 4a). $CD4^-CD8^-$ thymocytes (Fig. 4a, quadrant III, 18%), characterized in other species as thymic precursors, $CD4^+CD8^+$ (Fig. 4a, quadrant II, 53%) common thymocytes and single positive subpopulations either $CD4^-CD8^+$ (quadrant I, 13%) or $CD4^+CD8^-$ (quadrant IV, 16%) showing more mature phenotypes. As indicated in Fig. 2 for another individual, the majority of porcine thymocytes were $CD6^+$ (Fig. 2a, 90%, Fig. 4b, 94%). Only a small percentage of thymocytes was negative for CD6 (10% and 6%, respectively). Studies of the CD6 expression on the CD4/CD8 defined thymocyte subpopulations (Fig. 4a, c) revealed that the $CD6^-$ subset was enriched in the $CD4^-CD8^-$ thymic subpopulation (Fig. 4a, quadrant III, Fig. 4c, III, 20%) described for other species as thymic progenitors. All other thymic subpopulations, $CD4^+CD8^+$ common thymocytes (Fig. 4a, quadrant II, 53%) and more mature thymocytes either $CD4^-CD8^+$ (Fig. 4a, quadrant I, 13%) or $CD4^+CD8^-$ (Fig. 4a, quadrant IV, 16%) were positive for CD6 and showed minor percentages of $CD6^-$ cells (Fig. 4c, I, II, IV; 3%, 4% and 2%, respectively). Obvious was the lower CD6 antigen density on $CD4^+CD8^+$ thymocytes compared with the more mature single positive phenotypes (Fig. 4c, compare II with I and IV), which suggested that the CD6 expression on porcine thymocytes is up-regulated during thymocyte development.

Expression of wCD6 on extrathymic peripheral blood T lymphocytes

As shown in Fig. 1 the porcine CD6 antigen is exclusively expressed on T lymphocytes, but only a subset of T lymphocytes showed CD6 expression (Fig. 2b). To characterize the CD6 expression on CD4/CD8-defined T lymphocyte subpopulations, nylon-wool purified T lymphocytes were labelled with mAb against CD4, CD6 and CD8 antigens and analysed by three-colour FCM. The contour plot of the CD4 versus CD8 expression (Fig. 5a) demonstrated the labelling pattern characteristic for porcine PBTL. Besides $CD4^-CD8^+$(quadrant I, 47% in this individual) and $CD4^+CD8^-$ (quadrant IV, 12%) T-lymphocyte subpopulations, $CD4^-CD8^-$ (quadrant III, 33%) and $CD4^+CD8^+$ (quadrant II, 8%) T lymphocytes were prominent in the extrathymic T-lymphocyte population.

© 1996 Blackwell Science Ltd,

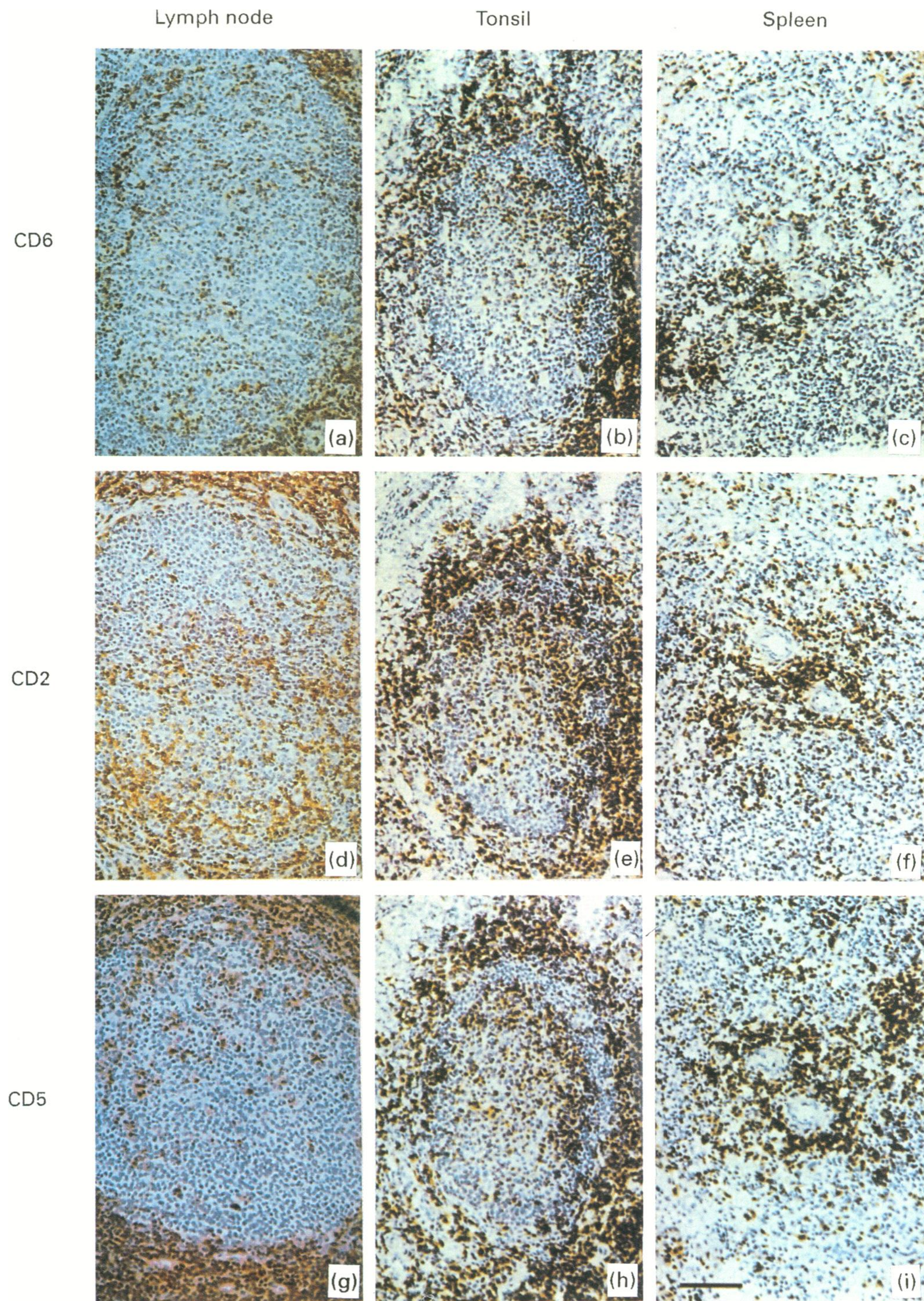

Figure 3. *In situ* localization of wCD6-positive T lymphocytes. Staining of cryostat sections of mesenteric lymph node (a, d, g), tonsils (b, e, h) and spleen (c, f, i) with mAb against the porcine CD6 (a–c), CD2 (d–f) and CD5 (g–h) differentiation antigens. The barr (i) indicates 0·1 mm.

Only 42% of the T lymphocytes of this animal were positive for CD6 (Fig. 5b). Analyses of the CD4/CD8-defined T-lymphocyte subpopulation for their CD6 expression revealed that all $CD4^+$ T-helper cells either $CD8^-$ (Fig. 5c, IV, 99%) or $CD8^+$ (Fig. 5c, II, 99%) showed a homogeneous CD6 expression. The $CD4^-CD8^+$ subpopulation of cytolytic T lymphocytes (Fig. 5a, quadrant I) was divided into two subsets (Fig. 5c, quadrant I) with the phenotypes $CD4^-CD6^+CD8^+$ (45%) and $CD4^-CD6^-CD8^+$ (55%). The $CD4^-CD8^-$ T-lymphocyte subpopulation (Fig. 5c, III), described as porcine TCR-$\gamma\delta$ bearing null cells[5] and characterized by specific antigens (e.g. SWC6[8]) showed a heterogeneous CD6 antigen density. The

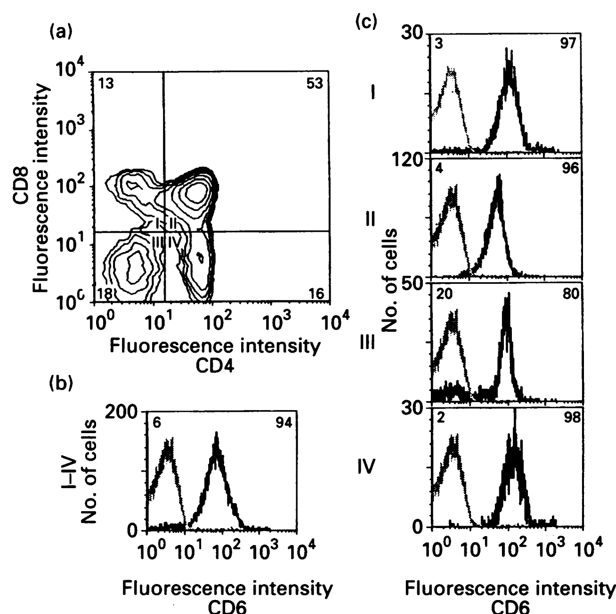

Figure 4. Expression of wCD6 on porcine thymocytes. Three-colour FCM analysis of porcine thymocytes labelled with mAb against CD4, CD6 and CD8. The fluorescence intensities of CD4-FITC and CD8-Cy5 are displayed as a two-dimensional contour plot (a) with contour lines representing levels of 20, 40, 60, 80, 100, 120 and 140 cells with a total number of 25 000 cells analysed. Quadrants I–IV are defined by background staining with fluorescent conjugates alone. Numbers in the corners indicate the percentages of cells in the respective quadrants. The CD6-PE fluorescence is depicted as line plot for all cells (b, quadrants I–IV) or for cells in the respective quadrants (c; I, II, III and IV, respectively).

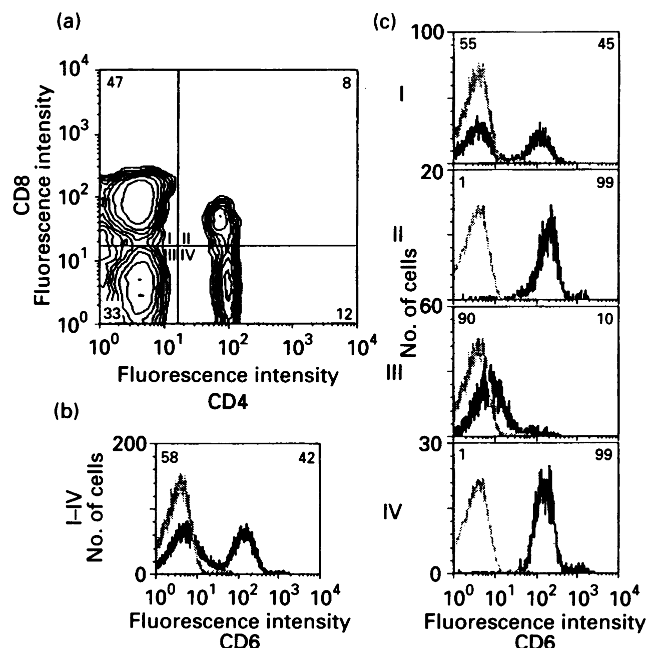

Figure 5. Expression of wCD6 on porcine peripheral blood T lymphocytes. Three-colour FCM analysis of CD4 (FITC), CD6 (PE) and CD8 (Cy5) expression on porcine peripheral blood T lymphocytes. The CD4 versus CD8 expression is shown as contour plot (a). The CD6-PE fluorescence is shown as line plot for all T lymphocytes (b) and the respective CD4/CD8-defined T-lymphocyte subpopulations (c; I, II, III and IV).

majority of the cells belonging to this subpopulation was negative for CD6 (90%), whereas some cells (10%) existed showing a very low CD6 antigen density.

This is in agreement with the results demonstrated in Fig. 2a, where all $CD2^-$ T lymphocytes, which had been characterized in peripheral blood as $CD4^-CD8^-$ T lymphocytes before[3,18] have been shown to be negative for CD6.

Functional characterization of wCD6-defined T-lymphocyte subsets

From earlier experiments it was known, that in the porcine T-lymphocyte population MHC-restricted T-cell specific functions could be assigned to the $CD4^+CD8^-$,[1] $CD4^+CD8^+$ (ref. 4 and A. Saalmüller unpublished data) and $CD4^-CD8^+$ [17] subpopulations. Whereas both $CD4^+CD8^-$ and $CD4^+CD8^+$ cells show MHC class II-restricted T-helper cell responses, MHC class I-restricted cytolytic T-cell activity could be distributed to the $CD4^-CD8^+$ T lymphocytes with high CD8 antigen density.[9] In contrast, non-MHC-restricted lymphocyte functions, e.g. spontaneous cytolytic activity against tumour cells, generation of lymphokine-activated killer cells (LAK) and the proliferative response against syngeneic leucocytes in mixed leucocyte reactions (MLR) could be either distributed to the $CD4^-CD8^{+low}$ subset[9,14] or to the $CD4^-CD8^-$ null cell population (A. Saalmüller, unpublished data). The CD4/CD8-defined phenotypes of the CD6-separated fractions and their respective functional *in vitro* cytolytic activities are shown in Fig. 6. Figure 6a demonstrates the CD4/CD8-defined T-lymphocyte subpopulations with the phenotypes $CD4^-CD8^+$ (Fig. 6a, quadrant I, 47%), $CD4^+CD8^+$ (quadrant II, 8%), $CD4^-CD8^-$ (quadrant III, 33%) and $CD4^+CD8^-$ (quadrant IV, 12%). Their CD6 expression is shown in Fig. 6b indicating 57% $CD6^-$ and 43% $CD6^+$ T lymphocytes. Separation of this population by electronic windows into the CD6-defined subsets showed an enrichment of $CD4^-CD8^-$ and $CD4^-CD8^{+low}$ T-lymphocyte subpopulations in the $CD6^-$ fraction (Fig. 6c, left contour plot), whereas the $CD6^+$ subset was represented by the $CD4^-CD8^{+high}$ and the $CD4^+$ T-lymphocyte subpopulations (Fig. 6c, right contour plot). To study the relationships between CD6 expression and *in vitro* functions, T lymphocytes were fractionated by magnetic cell separation into these defined subsets and used as effector cells in cytolytic assays. Cells with spontaneous cytolytic activity against K562 tumour cells (Fig. 6d) could be distributed to the $CD6^-$ T-lymphocyte fraction (Fig. 6d, third column, $CD6^-$). Compared to the non-separated control group (Fig. 6d, first column, unlabelled) and the mAb-labelled group prior to magnetic cell sorting (Fig. 6d, second column, mAb-labelled) an enrichment of effectors responsible for spontaneous cytolytic activity was visible. The $CD6^+$ T-lymphocyte fraction did not show any cytolytic reponse to xenogeneic target cells (Fig. 6d, fourth column, $CD6^+$). In contrast to the $CD6^-$ T-cell subset (Fig. 6e, third column), the $CD6^+$ cell fraction, separated from stimulated T lymphocytes after 1 week of *in vitro* cultivation with allogeneic PBMC, was able to recognize and lyse allo-

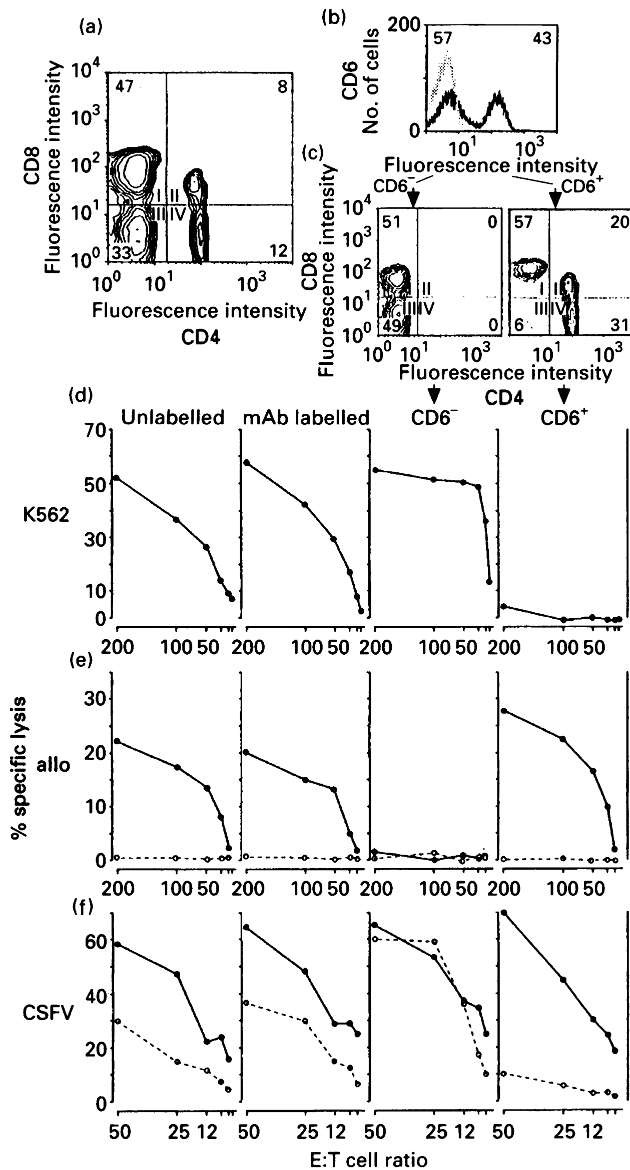

Figure 6. Cytolytic activities of wCD6-defined T-lymphocyte fractions. The CD4 versus CD8 antigen expression for all T lymphocytes analysed prior to CD6 separation is shown in (a) and their CD6 antigen expression in (b). The CD4/CD8 expression of the CD6-separated T-cell fractions is documented in (c). The cytolytic activities of T lymphocytes and the respective CD6-separated fractions: the spontaneous cytolytic activity of freshly isolated T lymphocytes against K562 tumour cells (d), the MHC-restricted cytolytic activity of alloantigen-stimulated T lymphocytes (e), and the virus-specific cytolytic activity of classical swine fever virus-specific T lymphocytes (f), was determined on ^{51}Cr-labelled target cells (d, K562; e, allogeneic blasts; f, CSFV-infected autologous cells). Unlabelled T lymphocytes (unlabelled) and T lymphocytes labelled with all antibodies necessary for the magnetic cell sorting (mAb-labelled) served as controls. Open circles show the lysis of syngeneic target cells (e) or non-infected autologous cells (f).

antigeneic target cells (Fig. 6e, fourth column). Obvious was an enrichment of MHC-restricted effector cells in the CD6$^+$ fraction compared with the non-separated control group (Fig. 6e, first column) and the mAb-labelled group (Fig. 6e, second column). Syngeneic target cells were neither lysed by CD6$^-$ nor by CD6$^+$ effector cells. T lymphocytes derived from classical swine fever virus (CSFV)-infected swine (Fig. 6f) and restimulated *in vitro* with viral antigens always showed besides the cytolytic activity against CSFV-infected target cells (Fig. 6f, unlabelled and mAb-labelled group, closed circles) high cytolytic activity against non-infected syngeneic cells (open circles). Separation into the CD6-defined T-lymphocyte fractions revealed a distribution of the non-specific cytolytic activity to the CD6$^-$ T-lymphocyte fraction (Fig. 6f, CD6$^-$), whereas the CD6$^+$ fraction was enriched for virus-specific cytolytic T lymphocytes showing no lytic activity on non-infected syngeneic target cells.

These results demonstrate that the expression of the putative CD6 can be used to discriminate between two subsets of porcine T lymphocytes, CD6$^-$ cells with non-MHC-restricted cytolytic activities and CD6$^+$ T lymphocytes with MHC-restricted antigen-specific functions.

DISCUSSION

A number of differentiation antigens had been reported in swine whose expression on mature T lymphocytes did not appear to be limited to particular T-cell subsets (e.g. CD2, CD5, SWC1). These antigens had been described as pan-T-cell markers. Even so expression of these antigens is not always restricted to mature T lymphocytes. For example, SWC1 is also expressed on myeloid cells,[20] CD2 is also expressed on natural killer (NK) cells[14] and CD5 can be found on most of TCR $\gamma\delta$ T lymphocytes and a subpopulation of B lymphocytes.[9] The expression of the 110 000 MW surface molecule recognized by the mAb a38b2 appears to be limited to thymocytes and a clear-defined subset of extrathymic T lymphocytes. B lymphocytes and cells of the myeloid lineage do not react with mAb a38b2. The apparent molecular mass and the tissue distribution of the antigen recognized by mAb a38b2 might specify this antigen as the porcine CD6 analogue. Sequence data of this molecule will contribute the definite proof. Therefore, according to the proposed nomenclature of the Second International wine CD workshop, the correct designation for this cluster including also other mAb (PG90A from Bill Davis, Pullman, WA[11] and MIL8 from Chris Stokes, Bristol, UK, the later one clustered in the Second International Swine CD workshop) would be wCD6.[12]

Although the function of CD6 antigen in the immune system is currently unknown, some reports suggest an involvement of the CD6 molecule in T-cell activation[27,28] in the major common pathway mediated through protein kinase C.[27] It has been also described that mAb directed against distinct epitopes of the human CD6 were capable of transducing activation signals to T cells. For two anti-CD6 mAb–anti-T12[29] and anti-2H1[30] — a comitogenic activity could be shown in T-cell activation in combination with costimulatory signals provided by accessory cells and phorbol myristate acetate, respectively. It has been suggested that the difference in epitope specificity of these two anti-CD6 mAb accounts for their distinct activation properties. In swine none of these functions could be demonstrated, indicating that mAb a38b2

recognizes an epitope on the putative porcine CD6 which is different from the epitopes characterized by the mAb against the human or bovine analogues. Recent cloning of the human CD6 cDNA demonstrated a high homology to the CD5 molecule, both surface antigens being characterized by three cysteine-rich domains and belonging to a new superfamily of proteins which includes the macrophage scavenger receptor type I and other peptide-binding receptors.[31] It can be speculated that CD6, which seems to be also in swine up-regulated during thymic development might be a receptor for thymic hormones responsible for maturation and differentiation of thymocytes to mature T lymphocytes. Based on the high homology to the CD5 it might be also possible that CD6 is involved in protein binding of CD72-like ligands, recognized by CD5.[32] But neither the binding to a putative ligand nor a presumable function in thymic development has been studied in swine.

In conclusion the described results indicate CD6 to represent an important molecule in the complicated pathways of T-cell differentiation and T-cell activation. But besides all these reports about the possible role of CD6 in the immune response and the activation of T lymphocytes, the CD6 expression on porcine lymphocytes enables a clear discrimination between antigen-specific and non-specific T lymphocytes which might belong to the TCR $\alpha\beta$ and the TCR $\gamma\delta$/ NK cell fractions, respectively.

ACKNOWLEDGMENTS

We thank Dr H.-J. Rziha and Dr M Büttner for critical reading of the manuscript.

REFERENCES

1. 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.
2. Saalmüller A., Reddehase M.J., Bühring H.-J., Jonjic S. & Koszinowski U.H. (1987) Simultaneous expression of CD4 and CD8 antigens by a substantial proportion of resting porcine T lymphocytes. *Eur J Immunol* **17**, 1297.
3. Saalmüller A. & Bryant J. (1994) Characteristics of porcine T lymphocytes and T-cell lines. *Vet Immunol Immunopathol* **43**, 45.
4. Pescovitz M.D., Sakopoulos A.G., Gaddy J.A., Hussman R.J. & Zuckermann F.A. (1994) Porcine peripheral blood CD4/CD8 dual expressing T cells. *Vet Immunol Immunopathol* **43**, 53.
5. Hirt W., Saalmüller A. & Reddehase M.J. (1990) Distinct $\gamma\delta$ T cell receptors define two subsets of circulating porcine $CD2^-CD4^-CD8^-$ T lymphocytes. *Eur J Immunol* **20**, 265.
6. Saalmüller 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.
7. Carr M., Howard C.J., Sopp P., Manser J.M. & Parsons K.R. (1994) Expression on porcine $\gamma\delta$ lymphocytes of a phylogenetically conserved surface antigen previously restricted in expression to ruminant $\gamma\delta$ T lymphocytes. *Immunology* **81**, 36.
8. Binns R.M. (1994) The null/ $\gamma\delta$ TCR^+ T cell family in the pig. *Vet Immunol Immunopathol* **43**, 69.
9. Saalmüller 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.
10. Saalmüller A., Aasted B., Canals A. *et al.* (1994) Summary of workshop findings for porcine T-lymphocyte antigens. *Vet Immunol Immunopathol* **43**, 219.
11. Saalmüller A., Aasted B., Canals A. *et al.* Analyses of mAb reactive with porcine CD6. *Vet Immunol Immunopathol* **43**, 243.
12. Saalmüller A. (1996) Characterization of swine leukocyte differentiation antigens. *Immunology Today*, in press
13. Pescovitz M.D., Lunney J.K. & Sachs D.H. (1984) Preparation and characterization of monoclonal antibodies reactive with porcine PBL. *J Immunol* **133**, 368.
14. Pescovitz M.D., Lowman M.A. & Sachs D.H. (1988) Expression of T-cell associated antigens by porcine natural killer cells. *Immunology* **65**, 265.
15. Hammmerberg C. & Schurig G.G. (1986) Characterization of monoclonal antibodies directed against swine leucocytes. *Vet Immunol Immunopathol* **11**, 107.
16. Lunney J.K. (1993) Characterization of swine leukocyte differentiation antigens. *Immunology Today* **14**, 147.
17. Jonjic S. & Koszinowski U.H. (1984) Monoclonal antibodies reactive with swine lymphocytes. I. Antibodies to membrane structures that define the cytolytic T lymphocyte subset in the swine. *J Immunol* **133**, 647.
18. Saalmüller A., Hirt W. & Reddehase M.J. (1989) Phenotypic discrimination between thymic and extrathymic $CD4^-CD8^-$ and $CD4^+CD8^+$ porcine T lymphocytes. *Eur J Immunol* **19**, 2011.
19. Saalmüller A. & Maurer S. (1994) Major histocompatibility antigen class II expressing resting porcine T lymphocytes are potent antigen presenting cells in mixed leukocyte culture. *Immunobiology* **190**, 23.
20. Saalmüller A., Reddehase M.J., Bühring H.-J., Jonjic S. & Koszinowski U.H. (1987) Monoclonal antibodies reactive with swine lymphocytes. II. Detection of an antigen on resting T cells down-regulated after activation. *J Immunol* **138**, 1852.
21. Pauly T., Elbers K., König M., Lengsfeld T., Saalmüller A. & Thiel H.-J. (1995) Classical Swine Fever Virus (CSFV)-specific cytotoxic T lymphocytes and identification of a T cell epitope. *J Gen Virol* **76**, 3039.
22. Binns R.M., Pallares V., Symons D.B.A. & Sibbons P. (1977) Effect of thymectomy on lymphocyte subpopulation in the pig. Demonstration of a thymus-dependent null cell. *Int Arch Allergy Appl Immunol* **55**, 96.
23. Hirt W., Saalmüller A. & Reddehase M.J. (1990). Distinct $\gamma\delta$ T cell receptors define two subsets of circulating porcine $CD2^-CD4^-CD8^-$ T lymphocytes. *Eur J Immunol* **20**, 265.
24. Binns R.M. (1982) Organisation of the lymphoreticular system and lymphocyte markers in the pig. *Vet Immunol Immunopathol* **3**, 95.
25. Lanier L.L., Allison J.P. & Phillips J.H. (1986). Correlation of cell surface antigen expression on human thymocytes by multi-color flow cytometric analysis: implications for differentiation. *J Immunol* **137**, 2501.
26. Scollay R., Bartlett P. & Shortman K. (1984). T cell development in the adult murine thymus: changes in the expression of the surface antigens Ly2, L3T4 and B2A2 during development from early precursor cells to emigrants. *Immunol Rev* **82**, 79.
27. Swack J.A., Mier J.W., Romain P.L., Hull S.R. & Rudd C.E. (1991). Biosynthesis and post-translational modification of CD6, a T cell signal-transducing molecule. *J Biol Chem* **266**, 7137.
28. Baldwin C.L., Machugh N.D., Ellis J.A., Naessens J., Newson J. & Morrison W.I. (1988). Monoclonal antibodies which react with bovine T-lymphocyte antigens and induce blastogenesis: tissue distribution and functional characteristics of the target antigens. *Immunology* **63**, 439.
29. Gangemi R.M.R., Swack J.A., Gaviria D.M. & Romain P.L. (1989). Anti-T12, an anti-CD6 monoclonal antibody, can activate human T lymphocytes. *J Immunol* **143**, 2439.

30. Mortimo C., Rudd C.E., Letvin N.L., Hagan M. & Schlossman S.F. (1988). 2H1-a novel antigen involved in T lymphocyte triggering. *J Immunol* **140**, 2165.
31. Aruffo A., Melnick M.B., Linsley P.S. & Seed B. (1991). The lymphocyte glycoprotein CD6 contains a repeated domain structure characteriztic of a new family of cell surface and secreted proteins. *J Exp Med* **174**, 949.
32. van de Velde V., von Hoegen I., Luo W., Parnes J.R. & Thielemans K. (1991) The B-cell surface protein CD72/Lyb-2 is the ligand for CD5. *Nature* **351**, 662.