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# Characterization of porcine T lymphocytes and their immune response against viral antigens

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**Abstract**

T lymphocytes play a central role in the antigen-specific immune response against various pathogens. To detect and to characterize porcine T lymphocytes, monoclonal antibodies (mAb) against leukocyte differentiation antigens had been raised and classified for their specificity. Analyses of porcine T lymphocytes with specific mAb against CD4 and CD8 differentiation antigens revealed differences in the composition of the porcine T-lymphocyte population compared to other species. In addition to the known subpopulations, $CD4^+CD8^-$ T helper cells and $CD4^-CD8^+$ cytolytic T lymphocytes, extra-thymic $CD4^+CD8^+$ T lymphocytes and a substantial proportion of $CD2^-CD4^-CD8^-$ T cell receptor (TcR)-$\gamma\delta^+$ T cells could be detected in swine. Functional analyses of porcine T-lymphocyte subpopulations revealed the existence of two T-helper cell fractions with the phenotype $CD4^+CD8^-$ and $CD4^+CD8^+$. Both were reactive in primary immune responses in vitro, whereas only cells derived from the $CD4^+CD8^+$ T-helper-cell subpopulation were able to respond to recall antigen in a secondary immune response. With regard to T lymphocytes with cytolytic activities, two subsets within the $CD4^-CD8^+$ T-cell subpopulation could be defined by the expression of CD6 differentiation antigens: $CD6^-$ cells which showed spontaneous cytolytic activity and $CD6^+$ MHC I-restricted cytolytic T lymphocytes including virus-specific cytolytic T lymphocytes. These results enable now a detailed view into the porcine T-cell population and the reactivity of specific T cells involved in the porcine immune response against pathogens. Furthermore this knowledge offers the possibility to investigate specific interactions of porcine T lymphocytes with virus-specific epitopes during vaccination and viral infections.


*Keywords:* Differentiation antigens; Porcine T lymphocytes; T-helper cells; Natural killer cells; Cytolytic T lymphocytes; Classical swine fever virus; Pseudorabies virus

## 1. Introduction

The importance of swine in agriculture has resulted in a substantial increase in research efforts during the past few years. Efforts to establish improved vaccination strategies, the design of

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more efficient vaccines and breeding for disease resistance, that might overcome the big economic losses associated with the disabling effects of viral, bacterial, and parasitic infections. These efforts were often based on a better knowledge of the porcine immune system. Following these reasons, studies on the porcine immune system have increased during the last years. In particular the use of monoclonal antibodies (mAb) which had been developed against distinct surface molecules of porcine leukocytes extended the information about porcine leukocyte populations and their interactions (Lunney, 1993; Saalmüller, 1996). These monoclonal antibodies have altered the way in studying the immune system as they allow the identification of surface molecules on immune cells, by which these cells can be classified, isolated and studied for their functional properties.

The knowledge about the porcine immune cell populations enables now comprehensive studies on the influence of infectious agents on the immune system and their interaction with distinct leukocyte populations (Susa et al., 1992; Martins et al., 1993; Martins and Leitao, 1994; Pauly et al., 1995, 1996). Understanding the interactions between immunogenic parts of infectious agents and the porcine immune cell populations is important for a further enhancement of the efficacy of existing and a targeted development of new vaccines against various pathogens.

To facilitate such developments, extensive research is necessary on the characterisation of antigen-specific reactions of B and T lymphocytes; furthermore there is particular need to elucidate the role of porcine T lymphocytes which contribute with their specificity, their variability and their immunological memory remarkably to the function of the antigen-specific immune response.

## 2. Phenotypical characterisation of porcine T lymphocytes

Within a diverse panel of T lymphocyte specific surface antigens, CD3 (cluster of differentiation 3) molecules represent the most potent marker for the characterisation and definition of this leukocyte population (reviewed in Clevers et al., 1988). T lymphocytes express together with their T-cell receptors (TcR), CD3 molecules, which are important for signal transduction and activation of the respective T lymphocyte clones (Kronenberg et al., 1986) after recognition of peptide antigens presented by molecules encoded in the major histocompatibility complex (MHC) (reviewed in Kronenberg et al., 1994), (Kronenberg et al., 1983; Hood et al., 1985; Allison and Lanier, 1987).

Due to the lack of monoclonal antibodies directed against CD3 that have not been described before 1996 (Saalmüller, 1996; Yang and Parkhouse, 1996; Yang et al., 1996; Pescovitz et al., 1998), porcine T lymphocytes were first characterised as thymus-derived cells expressing neither B-lymphocyte specific antigens nor myeloid cell markers (Saalmüller et al., 1987b; Saalmüller and Bryant, 1994).

### *2.1. Porcine T-lymphocyte subpopulations*

Leukocyte differentiation antigens (CD) of vertebrates have been highly conserved during evolution with regard to their molecular structure, their function and their tissue distribution. In consequence a large number of porcine leukocyte surface antigens represent analogous molecules of well defined differentiation antigens of other species.

Function, tissue distribution and sequence of the porcine CD2 and CD3 molecules seem to be similar to other species. The porcine CD2, a molecule of 48 kDa (Saalmüller et al., 1989), is able to bind xenogeneic sheep erythrocytes (Hammerberg and Schurig, 1986; Binns et al., 1988) and is also recognised by a murine antiserum directed against a highly conserved membrane region of the human CD2 analogue (Brown et al., 1988).

Some of the mAb directed against porcine CD3 were produced using the corresponding eukaryotic and prokaryotic expressed gene products as antigen (Kirkham et al., 1996; Yang et al., 1996). These mAb detect a protein with a molecular mass of 23 kDa and were able to stimulate porcine T lymphocytes (Kirkham et al., 1996; Pescovitz et al., 1998). Both differentiation anti-

gens CD2 and CD3 are indeed expressed on most of T lymphocyte population.

### 2.2. Expression of porcine CD4 and CD8

Porcine differentiation antigens CD4 and CD8 were characterised with regard to their molecular mass, their tissue distribution, and their functional behaviour and involvement as accessory molecules in the recognition of antigens presented by MHC molecules to the respective TcR. The porcine CD4 represents a monomeric glycoprotein of 55 kDa (Pescovitz et al., 1984, 1985, 1994a), which is expressed on about 50% of thymocytes and a substantial subset of extrathymic peripheral blood T lymphocytes. Monoclonal antibodies directed against the CD4a epitope (Saalmüller, 1996) are able to block MHC II-restricted functional activity of porcine T-helper cells (Pescovitz et al., 1985; Summerfield et al., 1996).

The porcine CD8 molecule is a dimeric molecule with a molecular mass of 70 kDa under non-reducing and 33–35 kDa under reducing conditions (Jonjic and Koszinowski, 1984; Pescovitz et al., 1984). CD8 molecules are exclusively expressed on a substantial proportion of thymocytes and a subset of T lymphocytes. Monoclonal antibodies directed against distinct porcine CD8 epitopes (Saalmüller et al., 1994a) are able to block MHC I-restricted antigen-specific cytolytic T-cell activity (Jonjic and Koszinowski, 1984; Pescovitz et al., 1985).

In thymus both antigens, CD4 and CD8 show an expression pattern comparable to that in other mammals (Saalmüller et al., 1987b, 1994b); besides $CD4^-CD8^-$ thymic progenitors and two fractions with the phenotype of more mature thymocytes, either $CD4^+CD8^-$ or $CD4^-CD8^+$, the majority of the porcine thymocytes belong to a population showing co-expression of the two differentiation antigens with the phenotype $CD4^+CD8^+$, which had been defined in other species as common thymocytes (Scollay et al., 1984; Lanier et al., 1986).

In the extra-thymic T-lymphocyte compartment the expression patterns of CD4 and CD8, presented in Fig. 1, differs completely from those known for other species (Pescovitz et al., 1985; Saalmüller et al., 1987b; Saalmüller and Bryant, 1994; Zuckermann and Gaskins, 1996). Besides $CD4^+CD8^-$ T lymphocytes (Fig. 1, quadrant IV, 19%) with the phenotype of MHC II-restricted T-helper cells and $CD4^-CD8^+$ T lymphocytes (Fig. 1, quadrant I, 43%) showing the phenotype of MHC I-restricted cytolytic T lymphocytes, two additional T lymphocyte subpopulations exist. Double-negative $CD4^-CD8^-$ T lymphocytes (Fig. 1, quadrant III, 17%), which have also been described for other ungulates, e.g. sheep (Mackay et al., 1986) and cattle (Mackay and Hein, 1990), and which in the blood-derived populations of pigs contain the majority of $CD2^-$ TcR-γδ T lymphocytes (Hirt et al., 1990; Saalmüller et al., 1990). An other subpopulation consist of a substantial proportion of $CD4^+CD8^+$ double positive extrathymic T lymphocytes (Fig. 1, quadrant II, 21%), which are unique for the porcine immune system (Pescovitz et al., 1985, 1994b; Saalmüller et al., 1987b).

These four subpopulations of extra-thymic T lymphocytes show high variance in their propor-

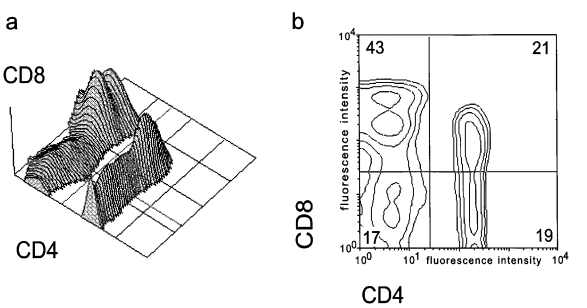


Fig. 1. Phenotypic characterisation of porcine T lymphocytes. Porcine peripheral blood T lymphocytes were labeled with mAb directed against CD4 (74-12-4, IgG2b), CD8 (295/33, IgG2a), and the respective immunoglobulin-isotype-specific conjugates; anti-mouse-IgG2b-FITC and anti-mouse-IgG2a-PE. The fluorescence intensities of 50 000 cells analysed are presented in: (a) as three-dimensional image; in (b) as two-dimensional contour plot, with CD4-FITC fluorescence intensity on the *x*-axis, and CD8-PE on the *y*-axis. Cells labeled with the conjugates alone define four quadrants (I–IV). The numbers in the corners represent the percentages of cells in the respective quadrants.

tion between different pigs (Saalmüller et al., 1987b) and the percentages of $CD4^-CD8^-$ and $CD4^+CD8^+$ seem to be inversely related (Saalmüller et al., 1987b) whereby the ratio of double-negative to double-positive T lymphocytes is age dependent: young animals show a high percentage of $CD4^-CD8^-$ T lymphocytes and only few $CD4^+CD8^+$ double positives; old animals are characterised by an inverse ratio (Saalmüller et al., 1987b). These data demonstrate postnatal, extra-thymic, antigen-dependent changes in the porcine CD8 expression on T lymphocytes, which is combined with a further maturation and development of the respective porcine T lymphocyte subpopulations. This differentiation could be simulated in vitro, showing a extra-thymic generation of $CD4^+CD8^+$ T lymphocytes upon activation (Saalmüller et al., 1987b, 1991; Zuckermann and Gaskins, 1996).

## 3. Functional characterisation of porcine T lymphocyte subpopulations

### *3.1. $CD4^-CD8^-$ T lymphocytes*

In the peripheral blood derived T-lymphocyte population, most of the $CD4^-CD8^-$ T lymphocytes are also negative for CD2 and express TcR-γδ chains (Saalmüller et al., 1989, 1990; Hirt et al., 1990, 1993; Davis et al., 1998). With regard to their surface antigen expression they represent a heterogeneous subpopulation (Reddehase et al., 1991; Binns et al., 1992, 1994; Hirt et al., 1993; Lunney, 1993; Thome et al., 1993, 1994; Binns, 1994; Carr et al., 1994; Saalmüller, 1996; Davis et al., 1998). The function of this subpopulation and the respective cell-subsets still has to be elucidated. Clear is that porcine TcR-γδ cells can be stimulated with mitogens and are able to show non-MHC-restricted cytolytic activity (Saalmüller, unpublished). There is yet no information about their in vivo functions, about the antigens recognised, the surface molecules involved in their functional capabilities and the phenotype of specific effector cells included in the heterogeneous subpopulation of $CD2^-CD4^-CD8^-$ TcR-γδ T lymphocytes.

### *3.2. $CD4^+CD8^-$ and $CD4^+CD8^+$ T lymphocytes*

In contrast to the $CD4^-CD8^-$ TcR-γδ T lymphocytes, the porcine CD4 subset, containing $CD4^+CD8^-$ as well as $CD4^+CD8^+$ T lymphocytes seems to be a more homogeneous T-lymphocyte population. All porcine CD4 positive T lymphocytes co-express CD2 (Saalmüller et al., 1989), CD3 (Yang and Parkhouse, 1996), CD5 (Saalmüller et al., 1994c,d), and CD6 (Saalmüller et al., 1994a) antigens. It seems that all $CD4^+$ cells belong to the TcR-αβ T lymphocyte subset. It should be mentioned, that porcine $CD4^+$ T lymphocytes can be discriminated by their CD8 expression into two subpopulations (Pescovitz et al., 1985; Saalmüller et al., 1987b). In contrast to all other species, a substantial proportion of CD4 positive extra-thymic T lymphocytes express CD8 antigens (Pescovitz et al., 1985; Saalmüller et al., 1987b) (Fig. 1, quadrant II); therefore two subpopulations of extra-thymic $CD4^+$ T lymphocytes exist: $CD4^+CD8^-$ cells with the phenotype of classical T-helper cells and $CD4^+CD8^+$ T lymphocytes which are unique for the porcine immune system. $CD4^+CD8^+$ extra-thymic T lymphocytes show indeed the phenotype of $CD4^+CD8^+$ common thymocytes but differ from thymocytes morphologically and phenotypically. With regard to the expression of other differentiation antigens $CD4^+CD8^+$ extra-thymic T lymphocytes show in contrast to $CD4^+CD8^+$ thymocytes, e.g. no expression of the thymocyte-specific CD1 antigen (Saalmüller et al., 1989; Pescovitz et al., 1990). Furthermore the extrathymic $CD4^+CD8^+$ T lymphocytes have the morphological phenotype of mature and resting T lymphocytes as shown in histology and electron microscopy (Saalmüller et al., 1991; Saalmüller and Bryant, 1994). Functionally are both $CD4^+$ T-lymphocyte subpopulations able to respond to mitogen and to alloantigen in mixed leukocyte cultures (MLC; Summerfield et al., 1996). All $CD4^+$ T lymphocytes are able to show T-helper-cell function for the generation of alloantigen-specific cytolytic T lymphocytes and T-cell dependent in vitro synthesis of immunoglobulins (Saalmüller, unpublished).

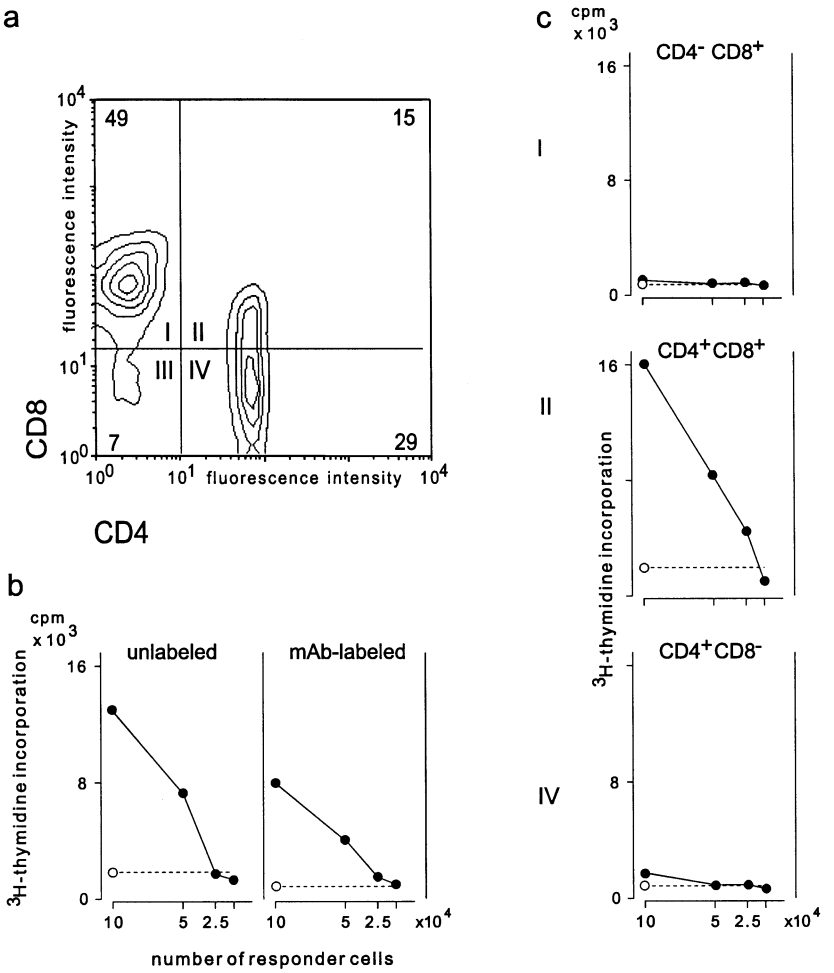


Fig. 2. Virus-specific proliferation of porcine T-lymphocyte subpopulations. T lymphocytes from a Pseudorabies-virus (PRV)-immunised pig and the respective CD4/CD8-defined, flow-cytometer (FCM)-separated T-lymphocyte subpopulations (c, I–III) were restimulated in vitro with UV-inactivated PRV, together with $1 \times 10^5$ autologous, irradiated peripheral blood mononuclear cells. (a) CD4 (FITC) versus CD8 (PE) expression of unseparated T lymphocytes. (b) PRV-specific proliferative response (solid circles), quantified by $^3$H-thymidine incorporation, of unseparated, unlabeled (unlabeled) and anti-CD4/anti-CD8-labeled (mAb-labeled) T lymphocytes. (c) PRV-specific proliferation (solid circles) of FCM-separated T-lymphocyte subpopulations (related to quadrants I, II, IV shown in (a)). Open circles show the proliferation of the respective subpopulations without in vitro restimulation with viral antigen. The figure was published previously by Summerfield et al. (1996).

In contrast to polyclonal stimulation with mitogen and oligoclonal activation in the MLC, which represent primary in vitro immune reactions, the two $CD4^+$ T-lymphocyte subpopulations differ completely regarding secondary antigen-specific responses against recall-antigen (Summerfield et al., 1996; Ober et al., 1998). Only $CD4^+CD8^+$ double positive T lymphocytes show a antigen-specific secondary immune response (Summerfield et al., 1996; Fig. 2, quadrant II). This reaction is MHC-class II restricted and the additionally expressed CD8 molecules seem to maintain no CD8-specific functional activity (Summerfield et al., 1996). These results demonstrate that in vivo generated virus antigen-specific memory T-helper cells belong to the $CD4^+CD8^+$ T-lymphocyte subpopulation.

### 3.3. $CD4^- CD8^+$ T lymphocytes

The porcine $CD4^- CD8^+$ T-lymphocyte subpopulation (Fig. 1, quadrant I) contains two main subsets of lymphocytes which can be discriminated by their immunological functions; one subset can be defined by spontaneous cytolytic activity against, e.g. xenogeneic tumor cells (Pescovitz et al., 1988); the other subset includes progenitors of MHC class I-restricted cytolytic T lymphocytes (Jonjic and Koszinowski, 1984). Cytolytic T lymphocytes (CTL) derived from these progenitor cells can be either directed against alloantigen-specific target cells after stimulation with alloantigen in MLC (Jonjic and Koszinowski, 1984; Saalmüller et al., 1987a, 1994d; Pauly et al., 1996) or in a secondary in vitro immune response against virus-infected autologous target cells (Martins et al., 1993; Pauly et al., 1995, 1996).

The $CD4^- CD8^+$ T-lymphocyte subpopulation is characterised by a heterogeneous CD8 antigen expression with very low up to high CD8 surface antigen density (Saalmüller et al., 1987b, 1994d; Pauly et al., 1996). Unique for all $CD8^+$ cells is their co-expression of CD2 molecules (Saalmüller et al., 1989; Saalmüller and Bryant, 1994). With regard to other differentiation antigens, e.g. the T-cell specific CD6 antigen (Saalmüller et al., 1994a; Pauly et al., 1996), the $CD8^+$ subpopulation can be divided into two subsets (Saalmüller et al., 1994a Pauly et al., 1996; Fig. 3(b, c)). $CD4^- CD8^+$ cells with low CD8 antigen density are negative for CD6 (Saalmüller et al., 1994a; Pauly et al., 1996; Fig. 3(b, c)), whereas $CD8^+$ cells with high CD8 antigen density show co-expression of CD6 molecules (Saalmüller et al., 1994a; Pauly et al., 1996). These features explain differences in the functional behaviour of these subsets: $CD3^- CD5^- CD6^- CD8^{low+}$ cells show spontaneous non-MHC-restricted cytolyic activity against tumour cells (Fig. 3(d); Pauly et al., 1996). CD8 positive cells with high CD8-expression include the progenitors of the alloantigen-specific cytolytic T lymphocytes (CTL; Fig. 3(e); Pauly et al., 1996) as well as virus-antigen specific CTL (Fig. 3(f); Pauly et al., 1995, 1996). The cytolytic activity of both types of CTL can be blocked by the addition of mAb directed against MHC class-I molecules and/or CD8a or CD8b epitopes (Jonjic and Koszinowski, 1984; Pescovitz et al., 1985; Saalmüller et al., 1994b). This confirms the functional difference to the $CD8^{low+}$ cell population and shows that the $CD3^+ CD5^+ CD6^+ CD8^{high+}$ T-cell fraction contains the MHC-class I-restricted cytolytic T-lymphocyte subset.

## 4. Porcine T-cell response against viral antigens

The number of studies about porcine cellular immune responses against viral pathogens (Chinsakchai and Molitor, 1994; Martins and Leitao, 1994; Saif et al., 1994) is still poor compared to that of other animal species. In the past most of the studies about interactions between pathogens and the porcine immune system could only suggest the participation of antigen-specific, MHC-restricted T lymphocytes in the respective immune responses and was discussed without final proof (Shimizu et al., 1977; Shimizu and Shimizu, 1979; Woods, 1979; Wardley, 1982).

T lymphocytes involved in anti-viral activities of the porcine immune system showing T-helper-cell activity were described to belong to the $CD4^+$ (Canals et al., 1992; Revilla et al., 1992;

Fig. 3. Cytolytic activity of CD8 positive T-lymphocyte subsets. (a) shows the CD4 versus CD8 expression of all T lymphocytes analysed prior CD6 separation and their CD6 expression. The CD4/CD8 expression of the CD6-separated T-cell fractions is documented in (c). The cytolytic activity of T lymphocytes (unlabeled, mAb-labeled) and the respective T-cell fractions ($CD6^-$ and $CD6^+$) is shown in (d–f): (d) demonstrates the spontaneous cytolytic activity of freshly isolated T lymphocytes against K562 tumour cells; (e) the MHC-restricted cytolytic activity of alloantigen-specific cytolytic T lymphocytes; and (f) the virus-specific reactivity of classical swine fever virus (CSFV)-specific T-lymphocytes. The cytolytic activity of the T lymphocytes and the respective fractions was determined on $^{51}Cr$-labeled K562 tumour cells (d), allogeneic blasts (e), and virus-infected autologous cells (f). Open circles show the lysis of syngeneic target cells (e) and non-infected autologous cells (f). This figure was published previously by Pauly et al. (1996).

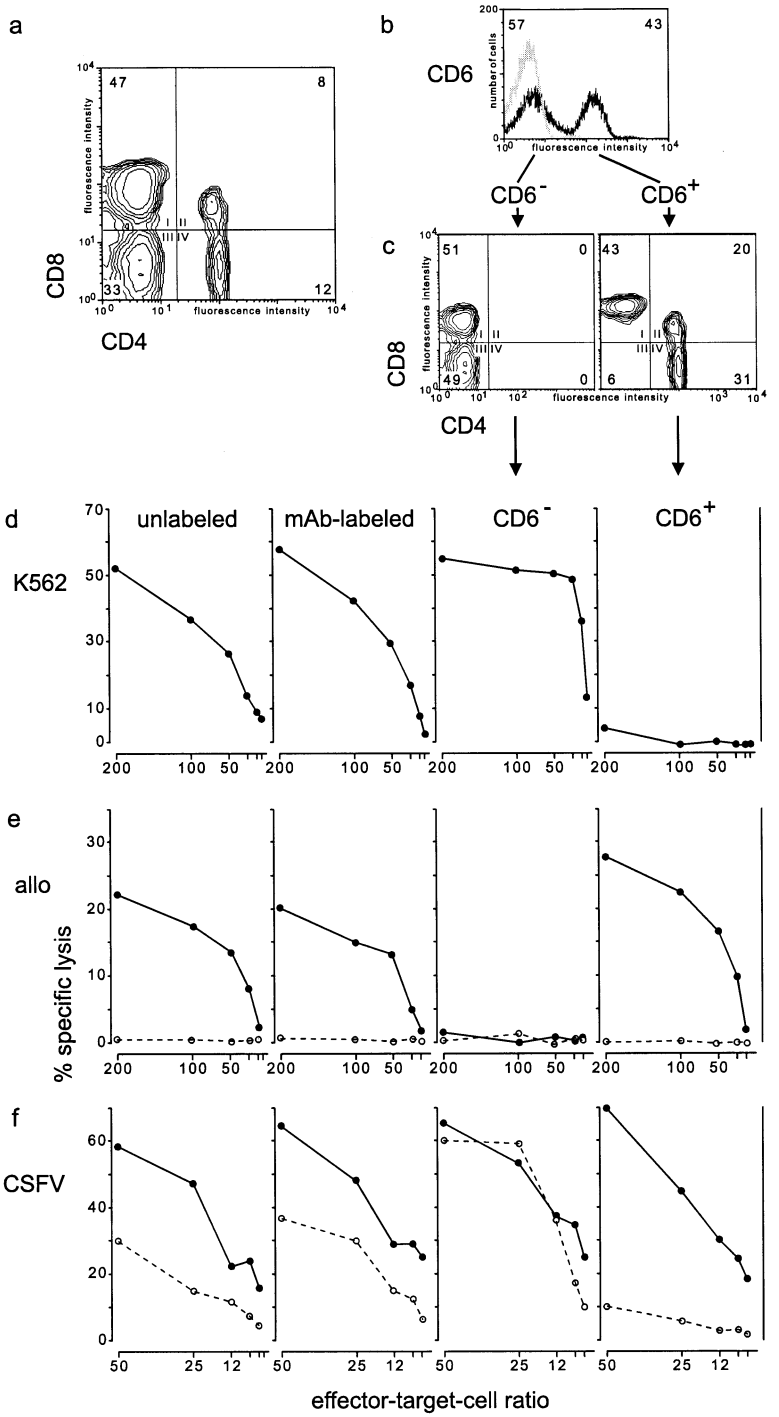
a
b
c
d
e
f
CD8
CD4
CD6
CD6-
CD6+
fluorescence intensity
number of cells
47
8
33
12
57
43
51
0
49
0
43
20
6
31
I
II
III
IV
unlabeled
mAb-labeled
K562
allo
CSFV
% specific lysis
effector-target-cell ratio

Fig. 3.

Table 1
Summary of the reactivity of porcine T lymphocytes against different viruses[a,b]

| Virus | ASFV | CSFV | FMDV | PRV | PRRSV | TGEV |
|---|---|---|---|---|---|---|
| *Reactivity of porcine T lymphocytes* | | | | | | |
| All T lymphocytes | | | | | | |
| Proliferation, blast transformation | + | + | + | + | + | + |
| T-helper cells | | | | | | |
| Cytokine-production help in Ig synthesis | + | na | + | + | na | + |
| | + | na | + | + | na | + |
| Cytolytic cell subsets | | | | | | |
| CTL | + | + | + | + | na | na |
| NK cells | + | na | na | + | na | na |
| Phenotype of the reactive T-cell population(s) | $CD4^+$, $CD8^+$, $CD4^+CD8^+$ | $CD4^+$, $CD8^+$ | $CD4^+$, $CD8^+$ | $CD4^+$, $CD8^+$, $CD4^+CD8^+$ | $CD4^+$ | $CD4^+$ |
| MHC-restriction of the anti-viral reaction | MHCI/ MHCII | MHCI/MHCII | MHCI/MHCII | MHCII | na | MHCII |
| Characterisation of T-cell epitopes | na | MHCI peptide | MHCII peptide | MHCII peptides | na | MHCII peptides |

[a] ASFV, African swine fever virus; CSFV, classical swine fever virus; FMDV, foot-and-mouth disease virus; PRRSV, porcine reproductive and respiratory syndrome virus; PRV, pseudorabies virus; TGEV, transmissible gasteroenteritis virus.

[b] The reactivity of the respective T lymphocytes, derived from infected or vaccinated animals, is indicated for: (a) all T lymphocytes, which show reactivity in proliferation or blast transformation assays; (b) for T-helper cells, by detection of their cytokine production or capacity in helping B lymphocytes in immunoglobulin synthesis; and (c) for cytolytic T lymphocytes, which are able to lyse virus-infected target cells in MHC-restricted (CTL) or non-MHC restricted manner (NK, LAK). The phenotype of the respective T-cell subpopulation as well as the MHC-restriction of the antigen recognition described so far are summarised. T-cell epitopes which have been characterised on the molecular level are described together with the phenotype of the respective restriction elements (MHCI or MHCII). References for the respective statements are included in the text. Groups with no information about the reactivity available were marked as na (not available).

Kimman et al., 1993; Rodriguez et al., 1994, 1995; Anton et al., 1995, 1996; Kimman et al., 1995) and $CD4^+CD8^+$ (Summerfield et al., 1996; Ober et al., 1998) double positive T-cell subpopulations. Regarding cytolytic-T lymphocytes the responding cells could be defined as $CD8^+$ MHC-class I-restricted cytolytic T lymphocytes (Zuckermann et al., 1990; Martins et al., 1993; Pauly et al., 1995, 1996) and $CD8^+$ cells with natural killer (NK) or lymphokine activated killer (LAK) cell activity (Kimman et al., 1996). Some interactions between viral antigens and the respective T-lymphocyte subpopulations has been characterised at least in MHC-defined inbred animals at the molecular level (Anton et al., 1995; Pauly et al., 1995). These experiments led to the identification of MHC-class I (Pauly et al., 1995) and MHC-class II restricted (Anton et al., 1995; Ober et al., 1998) viral T-cell epitopes. This information is summarised in Table 1.

On the other hand, these information, which might be important for more detailed analyses of the interactions between the porcine immune system and pathogens is still missing for the majority of porcine viruses.

The area of veterinary immunology concerning such molecular mechanisms of interaction between the porcine immune system and viral pathogens will be particularly important for future research aimed at controlling and eradicating a wide variety of infectious diseases of swine. These studies will facilitate the improvement of existing immune modulating drugs and vaccines and will also allow the design of new vaccines on the molecular level. All these activities will result in a better understanding of the porcine immune system at all, a better health of the animals, and a better quality of the products derived from these animals.

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