SHORT TERM MISSION REPORT

Federica Zuliani

Acquiring competence in cellular immunology diagnostics techinques

1. Name of applicant	Federica Zuliani
2. Contact information	Address: Via Tintoretto 11, Vigonza (PD), Italy Telephone: +39 3483234544 Fax: Email: federica.zuliani@gmail.com
3. Academic degrees	Degree in Veterinary Medicine
4. Current affiliation	Institute: Istituto Zooprofilattico Sperimentale delle Venezie, Viale dell'Università 10, Legnaro (PD) Department: SCT3
5. Position held (*Dottorando, borsista, contrattista a progetto, tirocinante*)	Scholarship holder
6. Name of the institute you plan to visit	Veterinärmedizinische Universität Wien, Institut für Immunologie, Department für Pathobiologie
7. Name and contact information of the scientist you plan to visit	Prof. Armin Saalmüller Veterinärmedizinische Universität Wien, Institut für Immunologie,Department für Pathobiologie. Veterinärplatz, 1 1210 Wien Austria T +43 1 25077-2750; +43 664 60257-2750 E-mail: armin.saalmueller@vetmeduni.ac.at
8. Duration and dates of the visit	4 weeks (from February 19th 2017 until March 19th 2017)

Introduction:

During the past, immunological diagnostics techniques were basically focused on the humoral branch of the immune system.

Vaccines efficacy is usually assessed by the detection of specific antibodies by means of ELISA tests. These tests are usually easy to perform and handle, commercially available and cheap. They can also distinguish among different classes of antibodies.

Their limit is that they give no information about the “quality” of antybodies: these simple surveys can reveal specific antibodies, but they can't really tell that there is an immunological protection, as the revealed antibodies cuold be not neutralizing (e.g.PRRSV, PCV2, M. Hyopneumoniae) and a correlation between clinical protection and the antibody titer does not always exist (Saalmüller, 2006).

Also, analysis of the only antibody titres is often not sufficient to evaluate a protective immune response to pathogens (Trible et al, 2012, Koinig et al, 2015).

Immune response to vaccination and to pathogens is based not only on antibody production, but there is also an involvement of the T-cells.

There are different methods to analyze a proctective and antigen-specific T cell-response.

These methods include the in vitro re-stimulation of the cells with appropriate antigens and than the analysis of: cellular proliferation (by means of fluorochromes), production of antigen-specific cytokines (e.g. interferon-γ ELISPOT), phenotypic charachterization of cells (flow cytometry).

ELISPOT is a sensitive assay used to identify cytokine-secreting cells at the single cell level. It can be used to investigate vaccine efficacy and for the characterization of cytotoxic T-cell activity by INF-γ, granzyme B and perforin analysis.

Flow cytometry analyzes both physical (relative size of cells and their granularity or complexity) and biochemical properties of cells .

Different immunological questions can be addressed by flow cytometry, regarding the phenotype of immune cells and its correlation to their functions (based on the expression of different CD molecules, see fig.1), distribution of cell phenotypes in different anatomic locations and the activation/differentiation status of immune cells, based on: cytokine prodiction, proliferation, apoptosis, live/dead discrimination, analysis of cytolytic activity of natural killer cells, CTLs, analysis of phagocytic activity.

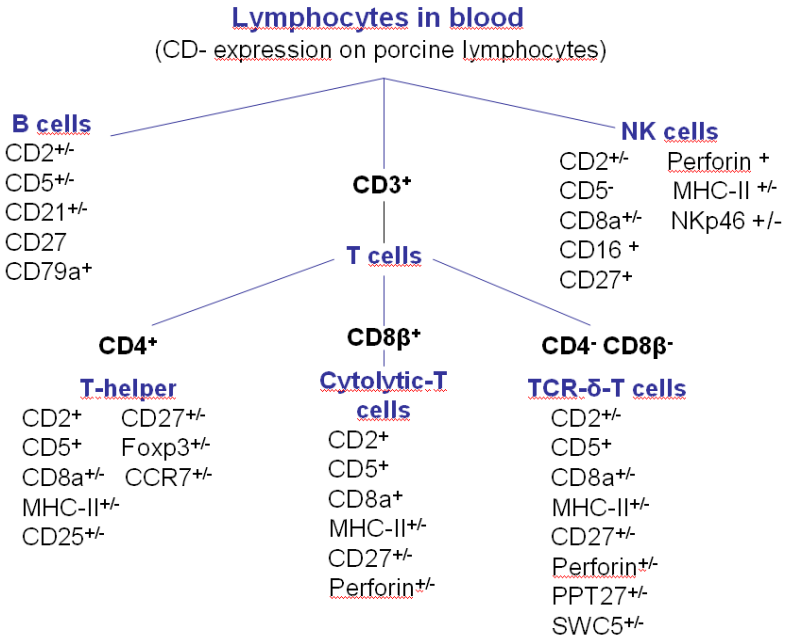

Fig. 1: multiple parameters that can be investigated by flow cytometry

Objectives:

The visit had the intent to give a global overview about the panel of diagnostic techniques available for evaluating the cell-immune response and to understand how to plan their use in some different situations, for example following up the evolution of an infection status toward a possible chronic illness or evaluating the protective cell-immune response after vaccination.

Moreover the purpose was also to create a link of veterinary skills including all the immunological aspects, to integrate the application of laboratory techniques for both humoral and cellular response.

At the same time, the visit to the University of Vienna, leader in the field of immunology, would be helpful to establish future collaborations, to constantly improve the techniques already used at the IZSVe.

The host institute

The University of Veterinary Medicine of Vienna (Vetmeduni), established in 1765, is the only academic educational and research institution in Austria that focuses on the veterinary sciences.

The Institut für Immunologie, was founded in 2004. Its staff conducts research and offers classes to deepen knowledge and the understanding of immune responses towards various pathogens. The institute has the goal to strengthen the veterinary immunology by intensive research into veterinary species, in close collaboration with the university clinic, focusing on both the detection of animals' immune responses and on the identification of the immune system's cells and their functional and phenotypic properties.

The researche focuses on the characterisation of the porcine immune system, with special reference to the innate and adaptive immune response.

The innate immune system includes :

- natural killer cells
- gamma/delta T cells

The adaptive immune system includes:

- T- helper cells
- cytolytic T cells
- regulatory T cells

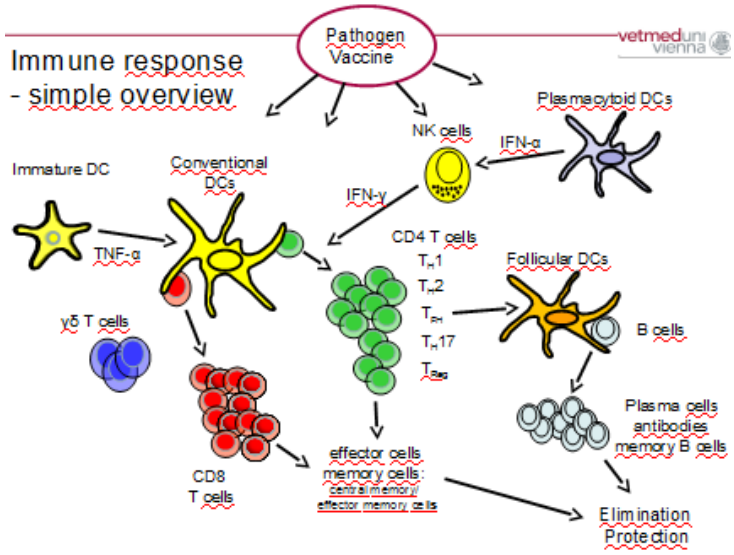

Fig. 2: Overview of t-cells involved in immune response. Courtesy of Prof. Saalmüller

The methods used are molecular, biochemical, cellular and immunological, like DNA / RNA analysis, PCR, cloning of gene fragments, immunoprecipitation, Western blotting, two-dimensional gel electrophoresis, generation of monoclonal antibodies, fluorochrome

conjugation of antibodies, flow cytometry, lymphocyte cultures, cell proliferation and cytotoxicity assays, ELISA and ELISPOT analysis.

Matherials and methods

The training was basedo on ELISPOT assay and Fluorcitometry, preceded by isolation of porcine peripheral blood mononuclear cells, stayning and proliferation of cells.

Isolation of porcine peripheral blood mononuclear cells (PBMC)

- Place 15 ml of lymphocyte separetium medium (LSM) into strile 50 ml tubes
- Estimate amount of blood and place the same volume of PBS into a sterile beaker
- Mix blood gently and pourinto prepared beaker and mix
- Carefully layer blood/PBS mixture using a 25 ml pipette over the lymphocyte separation madium and fill tubes to 50 ml
- Centrifuge 30 minutes at 920xg at 20°C
- Collect carefully the mononuclear cell layer with a 5 ml piprt and trasfer to a new 50 ml tube, previously cooled on ice and filled with 20 ml PBS and centrifuge 10 minutes at 470xg at 4°C.
- Remove supernatant, resuspend cells in residual PBS and fill up to 45-50 ml with PBS. Centrifuge as above
- Remove supernatant, resuspend cells in residual PBS and wash with 50 ml wash medium (RPMI 1650 5% FCS) and centrifuge as above
- Remove supernatant, resuspend cells in residual washing buffer and add 40 ml of cell culture medium.

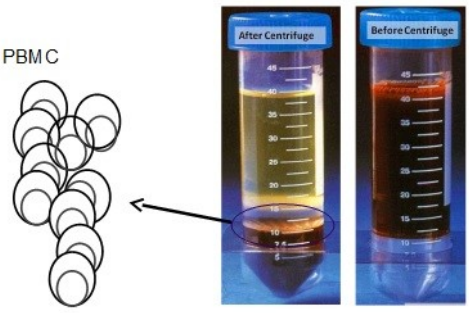

Fig.3.Collecion of PBMC

- To count the cells: put 10 µlof cell suspension in 90 µl of Turk's solution and mix well. Fill into a Neubauer counting chamber and calculate total number of isolated cells

Cell Trace Violet Proliferation Assay

- Defrost cells in PBS and count them, adjusting the number to 5-20 millions cells per ml of PBS

- Dissolve one vial of violet dye in 20 µl of dimethyl sulfoxide (DMSO)
- Diluite 5 µl of the solution of violet dye/DMSO wit 5 ml PBS and mix 1 ml of this solution with 1 ml of cell suspension and vortex it
- Incubate 10 minutes in a water bath at 37°C and vortex briefly several times
- Add 2 ml Fetal Calf Serum and incubate for 15 minutes, after vortexing, at room temperature and in the dark
- Add 10 ml cell colture medium (RPMI) and centrifuge (8 minutes- 1300 rpm, 20°C)
- Remove the supernatant, re-suspend in 14 ml of cell culture medium and centrifuge as above. Repeat this step for another time.
- Re-suspend in cell culture medium and count cells, then adjust them to reuired cell number and plate cells

T cell Stimulation

- Seeding the cells: $5x100^5$ cells per well in 100 µl in culture medium
- Add the stimuli: ConA 3 µg/ml final
  ConA 6 µg/ml final
- Incubate 4 days at 37°C

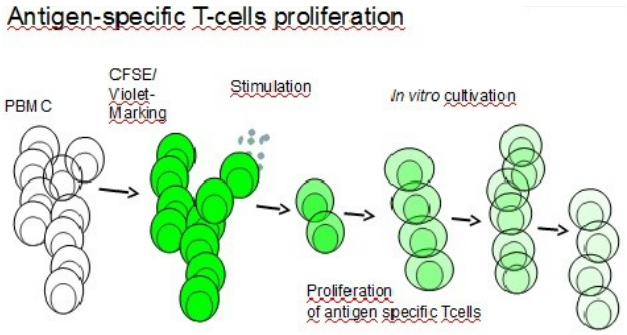

Fig. 4. Rappresentation of T-cells proliferation

Concanavalin A (ConA) is a lymphocyte mitogen, selective for T cells.

FCM staining

- Prepare cell suspension: $2x10^7$ /ml in staining buffer (PBS + 10% porcine plasma)
- Fill 20 µl cell suspension in 96-well round bottom microtiter plate (4x 10^5 cells per well)
- Add primary antibodies and controls and pulse-shake the plate

- Incubate for 20 minutes at 4°C
- Wash twice: add 200 µl/well staining buffer and centrifuge at 1500 rpm for 4 minutes at 4°C, discard the supernatant and wipe plate on absorbent paper quickly, then pulse-shake the plate
- Add secondary antibodies and controls and pulse-shake the plate
- Incubate for 20 minutes at 4°C
- Wash twice as above
- Resuspended cells in 250 µl of staining buffer
- Transfer cell in FCM tubes and analyse on flow cytometer

Fig 5. Flow cytometer, photo Vetmeduni

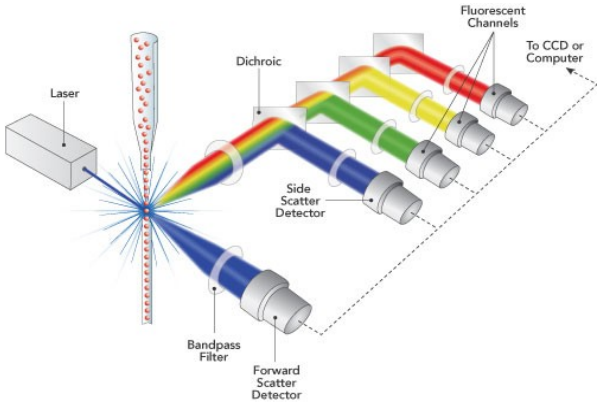

Fig 6. Principle of flow cytometry. Image from semrock.com

Voilet proliferation assay + T cell flow cytometry
buffer: PBS + 10% porc. Plasma OR PBS only
Staining in in MTP, analysis in FCM-tubes

No	primary ab	amount 1st	wash PBS	secondary ab	Amount/ dilution	wash porc. buffer	measuring in porc. buffer
1	C	w.o.	2x	w.o.		2x	250µl
2	CD4 (74-12-4) CD8α (11/295/33) CD3 (PPT3)	10µl 10µl 10µl	2x	anti-IgG2b-AFluor488 anti-IgG2a-AFluor647 anti-IgG1-PE VDeFluor780	1:600; 10µl 1:400; 10µl 1:200; 10µl 0,025µl	2x	250µl
3	TCR-γδ (PPT16) CD8α (11/295/33) CD8β (PPT23)	10µl 10µl 10µl	2x	anti-IgG2b-AFluor488 anti-IgG2a-AFluor647 anti-IgG1-PE VDeFluor780	1:600; 10µl 1:400; 10µl 1:200; 10µl 0,025µl	2x	250µl

all cells are stainined with Violet proliferation dye

Staining performed by:_____ **Date/CW of staining:**_____

PBMC used:_____ **Number of live cells / sample:**_____

Name of experiment in FACSDiva:_____

Fig.7. CD molecules investigated and related antibodies

Porcine INF-γ ELISPOT

Day 1. Work under sterile conditions

- Pre-wet the wells of a 96-well ELISPOT plate (Millipore MSIPS4510) with 30 µl of 35% ethanol for one minute and rinse with 150 µl sterile PBS three times
- Coat plates with 100 µl of coating antibody (mouse anti-swine INF-γ 0,5 mg/ml, Mabtech) diluted to 5 µg/ml in sterile PBS
- Wrap plate with cling film and store overnight at 4°C

Day 2. Work under sterile conditions

- Remove coating antibody solution and wash residual antibody with sterile PBS three times
- Block membrane with 200 µl/well of cell culture medium (RPMI-1640, 10% FCS) for at least an hour at 37°C
- Prepare cell-stimuli
  - ConA 3 µg/ml
  - ConA 6 µg/ml
  - IL-2 (20 ng/ml) + IL-12 (25 ng/ml) + IL-18 (50 gnml)
  - H3N2 virus (1.32x 10^8pfu): MOI 1.0
  - Mock: same amount as virus
- Cells were obtained from an unimmunized and uninfected swine and from a FLUAV immunized and infected swine, both from an animal trial performed at Immunology Institute.
- Resuspend the required cells in RPMI 1640 with 10% FCS, count the cells and adjust to required concentration (between 1x10^5 and 5x10^5 per well)
- Remove blocking medium and plate out cell stimuli and cell solution (each 100 µl/well).
- Incubate for 24 to 48 hours at 37°C

Day 3. Sterility is no longer needed

- Decant cells
- Wash the plate twice with distilled water and then three times with PBS+0,01% Tween 20
- Dilute the detection antibody (mouse anti-swine INF-γ, biotinylated, Mabtech) to 0,125 µg/ml in PBS/Tween/0,1% BSA and add it 100 µl/well
- Incubate the plate for 1 hour and 30 minutes at room temperature

- Wash the plate 4 times with PBS Tween
- Prepare streptavidin-alkaline phosphate enzyme conjugate 1:2000 dilution in PBS/Tween/0,1%BSA and add it 100 µl/well
- Incubate at room temperature for no longer than one hour
- Decant streptavidin and wash three times with PBS Tween and two times with PBS
- Add 100 µl/well of BCIP/NBT buffered substrate (Sigma) and incubate for no more than 5 minutes in the dark at room temperature.
- Stop spot development under running water and wash extensively. Remove plate underdrain and rise again
- Blot plate to remove residual liuid and dry back of wells thoroughly with an absorbent wipe.
- Let the plate dry in the dark.

Day 4.

- Analyze plate using AID imaging system

Results

Some representative example of flow cytometry and γ-interferon ELISPOT that have been carried out during the training are shown below.

Flow cytometry

Figure 8 shows the dimensions and complexity of the analised cells: not high intensity of both forward scatter light and side scatter light demonstrates that the lymphocytes are small cells and with low complexity/ granularity.

Figures 9 and 10 show the different T cells subsets on the basis of CD molecules.

In details:

- $CD4^+$- $CD3^+$: T-helper cells
- $CD4^+$- $CD8a^+$: Memory cells
- $CD8\beta^+$- $CD8a^+$: cytolytic T cells
- $CD8a^{+-}$- $TCR\gamma\delta^+$: γδ- T cells

Cellular proliferation can also be observed in peaks of proliferatig cells, with different brightness of violet signal: daughter cells, containing less dye, are less bright.

Two different concentration of the mytogen ConA were used, 3 µg/ml and 6 µg/ml, but no substantial difference in proliferation of T cell were observed.

APC	Allophycocyanin
FTTC	Fluorescin isothiocyanate
PE-A	Phycoerythrin

Tab.1. Fluorochromes associated with antibodies

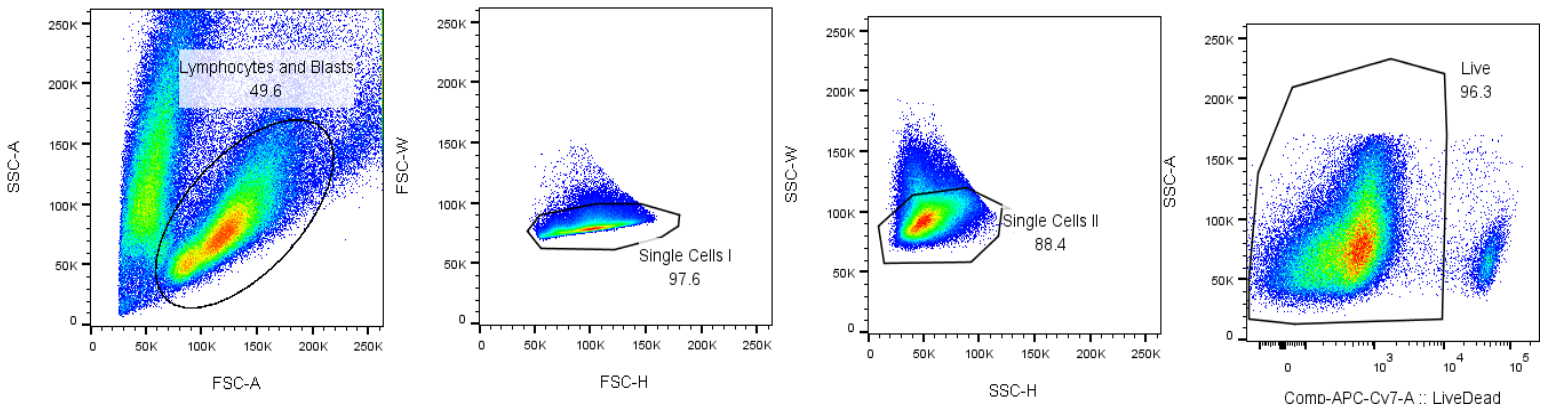

Fig.8. Visualisation of cells dimensions and complexity by means of forward scatteri light (FSC) and side scatteri light (SSC)

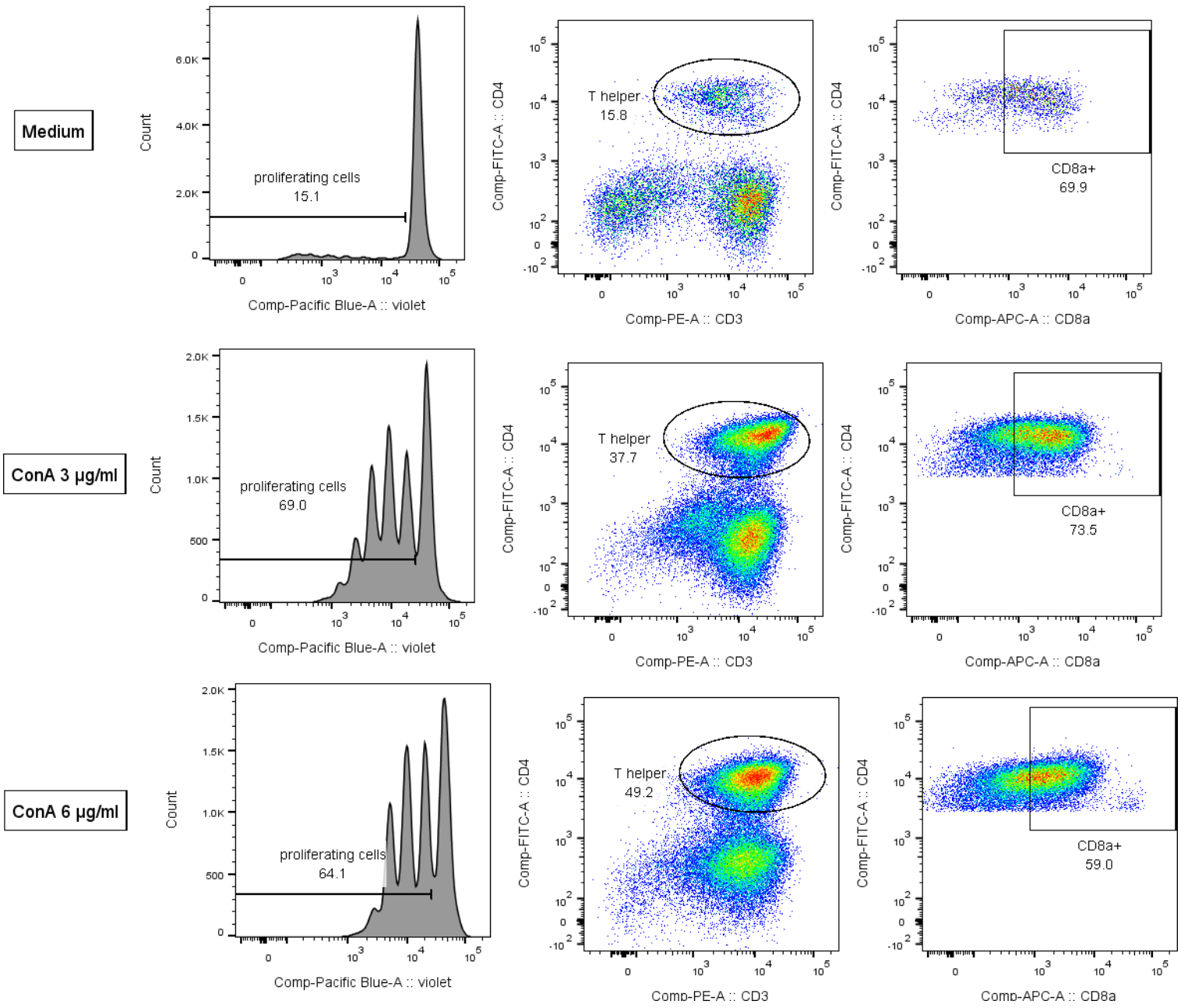

Fig.9. Visualisation of cells proliferation (on the right) and phenotype of t helper (in the middle) and memory cells (on the left) at different ConA concentration and without ConA (medium only)

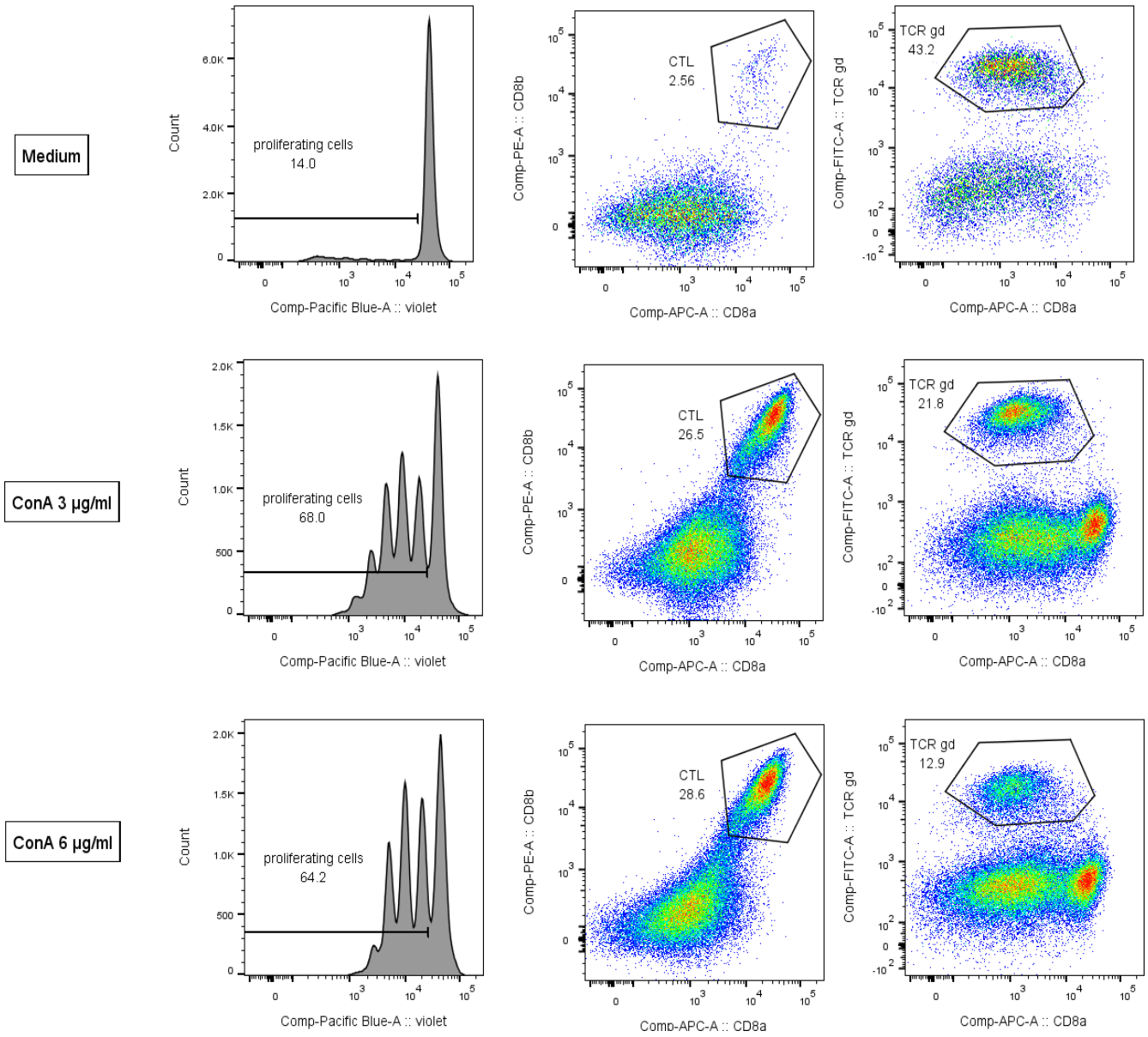

Fig.10. Visualisation of cells proliferation (on the right) and phenotype of cytolytic t cells (in the middle) andγδ-T cells (on the left) at different ConA concentration and without ConA (medium only).

IFN-γ ELISPOT

Table 2 and figure 12 show the different activity of stimulated T-cells of two swines of a trial of the Immunology Institute.

One is immunized and infected with H3N2 influenza A virus (FLUAV), while the other is unimmunized and uninfected. The number of γ-interferon producing cells is shown in the table, while in the plate's image the spots, representing each a single γ-interferon producing cell are visible.

It can be observed the higher number of activated cells of the immunized and infected swine confronted with the unimmunised and uninfected one.

The cells were also stimulated with ConA at two different concentrations (3 µg/ml and 6 µg/ml) and with a mix of IL-2, IL-12 and IL-18. Both ConA and cytokines are cells proliferation stimuli. In this tests they are used as positive controls.

Plate Data	1	2	3	4	5	6	7	8
A	0	3	4	13	23	28		
	0	19	28	117	165	342		
	-	-	-	-	-	-		
B	358	338	342	436	474	459		
	2265	2248	2184	2590	2909	2859		
	-	-	-	-	-	-		
C	227	164	166	263	306	324		
	1400	1164	1081	1560	1996	2140		
	-	-	-	-	-	-		
D	293	235	305	TNTC	178	179		
	2076	1678	2092	39863	1392	1473		
	-	-	-	-	-	-		
E	3	3	0	18	25	15		
	18	31	0	198	212	131		
	-	-	-	-	-	-		
F	10	3	6	131	109	176		
	40	7	35	979	878	1587		
	-	-	-	-	-	-		

- spots - activity (cytokine) - SFU

Tab. 2. Number of cell producing γ-interferon: A: only medium; B: cells stimulated with 6 µg/ml ConA; C: cells stimulated with 3 µg/m ConA, D: cells stimulated with IL-2+IL-12+IL18; E: mock control; F: cells stimulated with H3N2 virus.

From column 1 to 3: unimmunized and uninfected swine; from column 4 to 6: FLUAV immunized and infected swine.

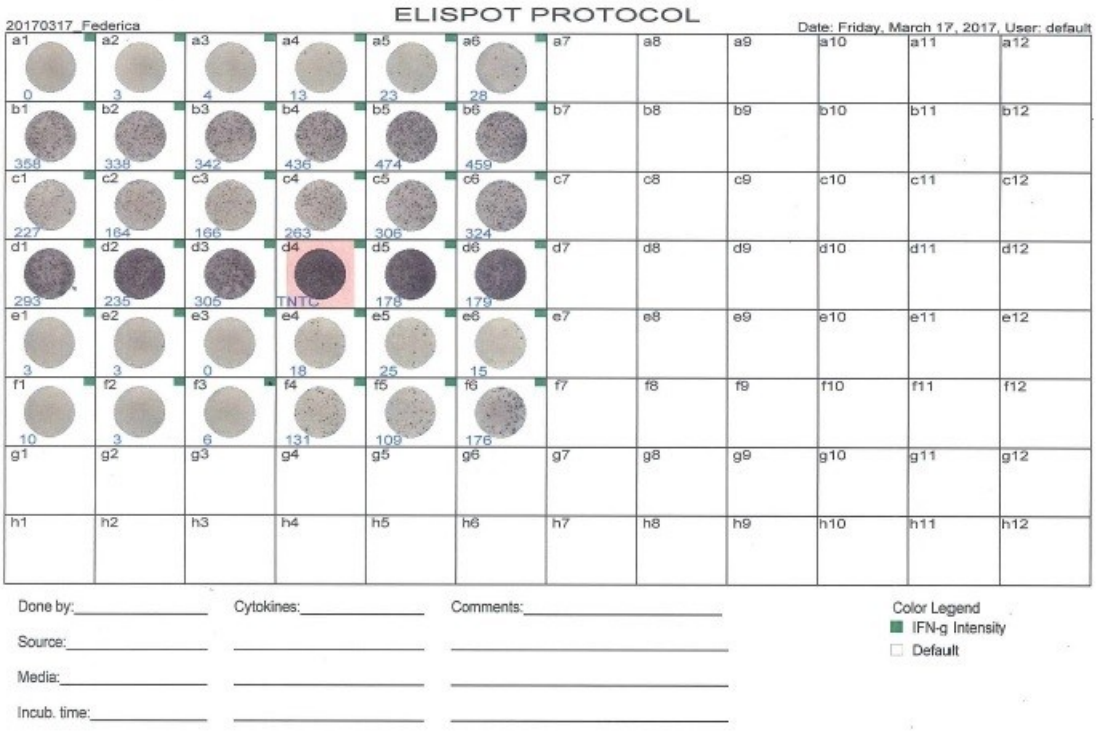

Fig.12. INF-γ plate, after reading with AID imaging system. A: only medium; B: cells stimulated with 6 µg/ml ConA; C: cells stimulated with 3 µg/m ConA, D: cells stimulated with IL-2+IL-12+IL18; E: mock control; F: cells stimulated with H3N2 virus.
From column 1 to 3: unimmunized and uninfected swine; from column 4 to 6: FLUAV immunized and infected swine.

Discussion and conclusions

The reactivity of specific T cells to pathogens or vaccines plays a very significant role in the understanding the immunological response to them.

Investigating cellular immunity helps to achieve more complete informations regardig the follow up of infectious diseases (Lunney et al., 2016).

In some diseases changes in t cells subsets can be correlated with the viral load (Lading et al., 2014, Talker et al., 2015) and in studies of vaccines effictiveness, changes in subsets and the production of certain type of cytokynes can predict the protective effect, better then the only antibody response analysis (Koinig et al., 2015).

The IFN-y ELISPOT represent a really sensitive method to measure the antigen (or vaccine)T cells reactivity. The limit of this method is that it does not provide any information about the t cells' subsets and their phenotype. This informations can be supply by the use of flow cytometry.

The right and complete knowledge and the understanding of immunological response after infection or vaccination allow the development of focused vaccines and strategies to deal with pathologies.

A combination of both B and T immune response should be investigate for each pathogen in order to define a really protective immunity.

Learning tha basis of these immunological techniques and improving their knowledge will permit me to integrate them with the serologic ones I already use during my work at Istituto Zooprofilattico Sperimentale delle Venezie, with the aim of create a link of veterinary skills that would include all the immunological aspects and to also evaluate the possibility to transfer those skills to other species (e.g. bovine) too.

References

- Koinig,H.K., Talker,S.C., Stadler,M., Ladinig,A., Graagr,R., Ritzmann,M., Henning-Pauka,I., Gerner,W. and Saalmüller,A., 2015. PCV2 vaccination induces IFN-γ/TNF-α co-producing T cells with a potential role in protection. *Veterinary research, 46:20.*
- Ladinig,A., Gerner,W., Saalmüller,A., Lunney,J.K., Ashley,C. and Harding,J., 2014. Changes in leukocyte subsets of pregnant gilts experimentally infected with porcine reproductive and respiratory syndrome virus and relationships with viral load and fetal outcome. *Veterinary research, 45:128.*
- Lunney,J., Fang,Y., Ladinig,A., Chen,N., Li,Y., Rowland,B. and Renukaradhya,G.J., 2016. Porcine reproductive and respiratory syndrome virus (PRRSV): pathogenesis and interaction with the immune system. *Annual review of animal biosciences, 4:129-154.*
- Saalmüller,A., 2006. New understanding of immunological mechanisms. *Veterinary microbiology, 117:32-38.*
- Talker,S.C., Koinig,H.C., Stadler,M., Graage,R., Klinger,E:, Ladinig,A., Mair,K.H., Hammer,S.E., Weissenböck,H., Dürrwald,R., Ritzmann,M., Saalmüller,A. and Gerner,W., 2015. Magnitude and kinetics of multifunctional $CD4^+$ and $CD8\beta^+$ Tcells in pigs infected with swine influenza A virus. *Veterinary research, 46:52.*
- Trible,B.R., Ramirez,A., Suddith,A., Fuller,A., Kerrigan,M., Hesse,R., Nietfeld,J., Guo,B., Thacker,E. and Rowland,R.R., 2012. Antibody responses following vaccination versus infection in a porcyne circovirus 2 (PCV2) disease model show distinct differences in virus neutralization and epitope recognition. *Vaccine, 30:4079-4085.*