Improving FIV detection in Peripheral Blood Mononuclear Cells using Dendritic Cells

Report of a research project by B. Joha
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Summary

Feline Immunodeficiency Virus (FIV) replicates in T-lymphocytes, more specifically in the peripheral $CD134^{+}$ subset, resulting in a diminished general resistance against common pathogens.
Dendritic cells (dc's) are antigen presenting cells collecting antigen at various places in the body and offering this antigen selectively to naive T-cells in lymph nodes.
This research examined possible enhancement of T-lymphocyte infection using dendritic cells resulting in a more rapid detection of FIV antigen in an attempt to speed up the diagnostic process. Thymocytes and peripheral blood mononuclear cells (PBMC's) were combined and cultured with FIV and/or dc's. A semi quantitative p24 ELISA was used to measure the degree of infection. Thymocytic cell infection appears to be enhanced by dc's whereas the influence of dc's on FIV infection in PBMC's did not show a clear effect. Concanavalin A appears to result in an enhancement of the production of FIV in PBMC's.

Introduction

Dendritic cells

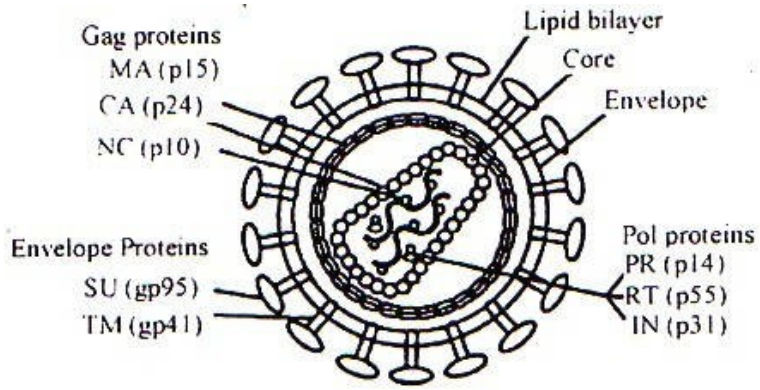
Dendritic cells (dc's) are derived from $CD34^+$ myeloid bone marrow progenitor cells which differentiate under influence of Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF) into immature dendritic cells. Through the vascular system they migrate towards organs and peripheral regions of the body. Part of these immature dc's reside in the blood being part of the peripheral blood mononuclear cell population whereas other dc's can be found in epithelial surfaces like skin and mucosae. In epithelial surfaces a specialised kind of dendritic cells is present called Langerhans cells; these cells can also be found in organs, e.g. spleen, kidney and heart. They are the only cell type in the epidermis that expresses MHC II molecules (Lotze & Thomson, 1999).
Immature dc's express large amounts of adhesion molecules (C-type lectins (e.g. DC-SIGN and Toll-like receptors (Van Kooyk et al., 2003)) and receptor molecules like CD205 by means of which the dc's perform phagocytosis of antigens and are capable of direct uptake of antigens by means of macropinocytosis (Janeway et al., 2005). Viewed by means of a light microscope it is possible to see dc processes bulging in and out from the surface: an indication of uptake activity.

If triggered by inflammatory cytokines dc's migrate through the lymphatic system towards local lymph nodes, a process during which they mature. Their phagocytic and macropinocytic capability disappears, instead they present on their surface large amounts of MHC-II and/or MHC I and a diverse set of adhesion and co-stimulatory molecules (e.g. B7). Besides they secrete cytokines (DC-Chemokine (DC-CK), IL-12, IL-18 and interferon-γ (IFN-γ) which are specifically used to attract naive T-cells (Janeway et al., 2005).

FIV

FIV is a lentivirus and is as such a member of the retroviridae family, subfamily orthoretrovirinae. The virus has an elliptical form, measures 80-100nm in diameter and is surrounded by an outer envelope with spiked surface projections. Inside this envelope a capsid is located containing two single-stranded strands of RNA (Bendinelli et al., 1995).

Picture 1: FIV (origin: Egberink (1991))



Prevalence of FIV is estimated at 1% to 14% in cats with no clinical signs, ranging up to 44% of sick cats (Amioka et al., 1998). Infection usually occurs after the first year of life. Transmission of the virus is largely due to bite wounds; seropositivity is reported twice as much in tomcats in comparison to queens as a result of differences in social behaviour. Presence of the virus in sputum has been detected in naturally infected as well as in experimentally infected animals. Infection through mucosae and placentae has been reported (Bendinelli et al., 1995 and Egberink, 1991, p. 19).

The clinical effects of a FIV infection manifest themselves as a diverse pattern of infections with opportunistic pathogens in a variety of organs. The virus compromizes the immune system by decreasing the amount of peripheral CD4+ T lymphocytes. When CD4+ T lymphocytes counts fall (in HIV starting below 500 cells per microliter) this impairs the activation of macrophages and B cells leading to a diminished immune response to pathogens (Janeway et al., 2005).

Following their entrance into the body viral particles are ingested by antigen presenting cells (apcs), mainly dc's. Unlike most viral infections where viral particles are carried inside endosomes which fuse with lysosomes, presenting the resulting viral debris at the surface of the apc, HIV, and probably FIV as well, escapes this mechanism. Endosomes carrying HIV particles will not fuse with lysosomes and are thought to survive for prolonged periods due to this mechanism (Van Kooyk & Geijtenbeek, 2003 and Janeway et al., 2005). However they do migrate towards the cell membrane where the viral particles are eventually presented at the surface of the apc's attached to MHC-II molecules. Here a direct adhesion between the dc and the T cell occurs. This process in turn promotes the up regulation of co-stimulatory and other molecules like B7 (Lotze & Thomson, 1999). In the end this results in T-cells being infected whereas the apc usually will not get infected and will not initiate viral replication although findings show that high concentrations of HIV *can* allow viral infection of dc's, resulting in the production of HIV by dc's

(Van Kooyk & Geytenbeek, 2003). There are also indications that simian immunodeficiency virus can infect dc's (Izmailova et al., 2003).

Presence of FIV in the body can be demonstrated by detecting antibodies against (parts of) the virus (in-house tests, e.g. Snap test™ (IDEXX corp.), immunofluorescence tests), by detecting the p24 antigen of FIV itself or by isolating FIV. Confirmation of viral presence can be provided by western blotting, in this test antibodies attach to FIV specific proteins (Bendinelli et al., 1995).

Research Goal

At present confirmation of the presence of FIV in cats using a p24 ELISA after PBMC culture takes two weeks up till two months. Findings show that HIV antigen detecting ELISAs can be sped up by adding dendritic cells to cultures of T-lymfocytes (Cameron et al., 1992). To examine if the same results can be achieved with FIV and dendritic cells this research aims to examine the influence of adding dendritic cells to T-cells, more specifically peripheral blood mononuclear cells from infected FIV cats, on viral replication in these cells.

Materials and Methods

All culturing was performed in Iscoves' Modified Dulbecco's Medium with Glutamax™ (Gibco/Invitrogen, Cat.No. 041-90898 M). This medium was completed by adding the following:

- 10% inactivated (by warming for 30 minutes at 56 °C) Fetal Bovine Serum (Hyclone, Cat. No. SH30071.03; Lot. No. ALM15036)
- 100 IU Penicillin/ml
- 100 IU Streptomycin/ml
- 50mM 2-Mercapto-Ethanol in MilliQ water

  (2-Mercapto-Ethanol is used to reduce disulfide bonds for electrophoretic analysis of protein. At low concentrations, mercapto ethanol prevents the oxidation of proteins)

This medium is in this text referred to as Iscoves' complete medium.

Dendritic cell culturing.

The culturing of dc's takes place in Iscoves' complete medium with addition of

- 0.01µg/ml rFeline Granulocyte-Macrophage Colony Stimulating Factor
- 0.01µg/ml rFeline Interleukin-4 (r&D, 984-FL 10µg/ml in $PBS^{Mg/Ca}$ + 0.1% BSA)

Granulocyte Macrophage Colony Stimulating Factor (GM-CSF) is a type specific stimulator of bone marrow cells. It stimulates the development of bone marrow derived progenitor hematopoietic cells into granulocytes, macrophages and eosinophils (Janeway et al., 2005 and Bienzle et al., 2003).

Interleukin-4 (IL-4) stimulates the proliferation, differentiation and activation of different cell types including fibroblasts, endothelial cells and epithelial cells. It prohibits the differentiation of myeloid progenitor cells into macrophages and monocytes but promotes the development of these progenitor cells into (immature) dendritic cells.
(Talmor et al., 1998; Bender et al., 1996)

BMMCs

Bone marrow derived mononuclear cells (BMMCs), derived from a specific pathogen free (SPF – FIV free) cat (Virology department internal referring number 80) were thawed, resuspended in Iscoves' complete medium, centrifuged and washed with fresh Iscoves' complete medium in order to remove the dimethyl sulfoxide (DMSO) medium which was used to freeze

the samples. Eventually these cells were resuspended in Iscoves' complete medium with addition of GM-CSF and IL-4 as mentioned above. When performing the first experiments of a series the BMMCs were placed in the first well of a six-well plate dish (Greiner). After two days the contents of this well was transferred into the adjacent well. At day four the same procedure was repeated by transferring the contents of the second well into the third one, adding fresh medium and cytokines. This procedure assured that monocytes could adhere to the bottom of the well and stay behind in the first and second well. After six days of culturing the BMMCs developed into immature dendritic cells. Monitoring of the result was performed by means of a light microscope.
After several BMMC cultures produced following the above mentioned protocol the procedure was changed: the washed BMMCs were transferred into a 100mm petri dish (Corning Incorporated, Cat. No. 430293) where they resided for at least six days. This procedure yielded an equal amount of dc's as the former and the development into macrophages proved to be sufficiently suppressed.

PBMC's

Peripheral blood mononuclear cells (PBMC's) were thawed and washed in an identical way as the BMMC's but were subsequently resuspended in Iscoves' complete medium with or without addition of Concanavalin A conform the protocol used with thymocytic cells. The PBMC's were incubated overnight at 37°C with 5% CO_2 in 100mm petri dishes instead of 6-well plate dishes in order to prevent cell adhesion. Furthermore, after the first batch of PBMC's was thawed it appeared that PBMC's are rather fragile: handling these cells by pipetting them too fast resulted in a large amount of cell debris.
When cultured in medium containing Concanavalin A the PBMC's were washed after two days in order to remove the old medium and were resuspended in a fresh Interleukin-2 (IL-2) medium (Chiron BV. Proleukin, r hu IL-2, 200 IU/ml). After one or two days culturing in IL-2 medium an experiment was started.

In the first series of experiments SPF thymocytic cells were infected with FIV. These thymocytes originated from feline thymocytes (isolated from a kitten in September 1993 at the department of Virology) being continuously in culture since October 2003. The thymocytes were stimulated every second week with Con A (5 mg/ml). Con A is a plant lectin which mimics antigen stimulation of T cells resulting in cell division. After two days of stimulation thymocytes were centrifuged, washed and resuspended in Iscoves' complete medium with addition of IL-2.

When infecting FIV-negative thymocytes or PBMC's with virus dilutions, free virus was used. This virus was originally cultured on thymocytes

(Department of Virology AV-stock Thymo-113). In every successive experiment a new virus sample was taken from the -80ºC freezer, thawed on ice and diluted into the desired dilutions in Iscoves' complete medium.

ELISA protocol

CELISA FIV: Antigen Detection

Introduction: One monoclonal Antibody is attached to an ELISA plate. Virus containing culture supernatant is lysed and added. The amount of p24 bound is detected by an biotinylated Monoclonal Antibody which recognise an other epitope. Avadinperoxidase has 4 binding sites for Biotin. The OD is proportional with the amount of p24. Detection limit 0.2ng based on whole viral protein.

Materials:

- Microtiterplate: ELISA plate (Greiner, catno: 655092)
- PBS with Mg and Ca.
- PBS without Mg and Ca (Biowhittaker, catno: 17-512F)
- PBS without Mg and Ca + 0.05% Tween 20 (Merck, catno: 822184)
- Blocking buffer: PBS without Mg and Ca + 0.05% Tween 20+ 5% NBS.
- NBS: New Borne Serum
- Substrate: prepare prior to use
    - Milli Q water 10.0ml
    - 1M sodiumacetate citric acid pH5.5 1.1ml
    - 3% Hydrogen peroxide 22µl
    - 6 mg/ml TMB in DMSO 183µl
- TMB: Tetramethylbenzidine
- Stopsolution: 2M H_2SO_4
- MA: 11C7C7C1 (23-12-1993)
- Ma conjugate: 5E6D11 biotin (25-02-1994)
  Stock (=60 times diluted with 50% glycerol, this means you must dilute 2500x instead of 50000x.)
- Extravidin Peroxidase (Sigma, catno: E2886)
- ELISA reader: warming up time 30 minutes.

Method:

1. Coating: 100µl 2000x diluted MA 11C7C7C1 in PBS with Mg and Ca overnight 4° C.
2. Wait until the plate has reached room temperature. Wash plate in ELISA plate washer at program 13 (ARNO) with bidest.
3. Blocking: 200µl Blockingbuffer, 1 hour at 37° C.
4. Wash plate in ELISA plate washer at program 13 (ARNO) with bidest.
5. Antigen: 100µl culture supernatant, lysed 15 minutes with 0.1% TritonX100 (=135µl + 15µl 1% Triton X100, 15min RT.) $1^1/_2$ hour at 37 C.
6. Wash plate in ELISA plate washer at program 13 (ARNO) with bidest.
7. MA conjugate: 100µl 2500x diluted in Blockingbuffer, 1 hour at 37° C.
8. Wash plate in ELISA plate washer at program 13 (ARNO) with bidest.
9. ExtravidinHRP: 100µl 3000x diluted in Blockingbuffer, 1 hour at 37 C.
10. Wash plate in ELISA plate washer at program 13 (ARNO) with bidest.
11. Substrate: 100µl incubation between 10 and 60 minutes at RT. Depending on the velocity of colour development of a positive control sample.
12. Stopreaction: add 100µl 2M H_2SO_4.
13. Shake plate, measure optical density at 450nm.

Results

Experiments

1.
As a pilot study SPF thymocytes were infected with increasing dilutions of FIV. Aim of this pilot study was to examine if and to which extent infections of thymocytes with FIV would occur.
48 wells of a 96 well round bottom cell culture plate (Corning Inc., Cat. No. 3799) were filled with $5x10^4$ thymocytes per well in 50µl Iscoves' complete medium. 24 Of the wells were filled with 8 different FIV dilutions in triplo in 50µl Iscoves' complete medium. 100µl Iscoves' complete medium was added to these wells. The remaining 24 wells were used as mock and filled with 150µl Iscoves' complete medium. For the layout of the plate see Diagram 1 below:

Diagram 1: Setup pilot experiment Thymocytes +/- FIV-titration

FIV Dilution	Thymocytes / FIV in triplo			Thymocytes / Mock in triplo		
not diluted						
5x						
25x						
125x						
625x						
3125x						
15625x						
78125x						

50 µl $5x10^4$ thymocytes
50 µl FIV-dilution
100 µl Iscoves' complete medium

50 µl $5x10^4$ thymocytes
150 µl Iscoves' complete medium

The 96 well plate was incubated for two hours at 37°C. Following incubation the plate was centrifuged twice at 1200 rpm for 10 minutes and washed with Iscoves' complete medium. Eventually they were refilled with 200 µl Iscoves' complete medium with IL-2. The plate was then placed in an incubator with CO_2 supply at 37°C for six days. After this

period 135µl of supernatant per well was transferred into a new 96 well plate. This supernatant was used in a p24 ELISA. The results were analysed using Students' T-test, Two-Sample Assuming Equal Variances. This analysis showed a significant difference between the groups 'thymocytes with FIV' and 'thymocytes without FIV' in the category 'no dilution':

Figure 1: Thymotitration

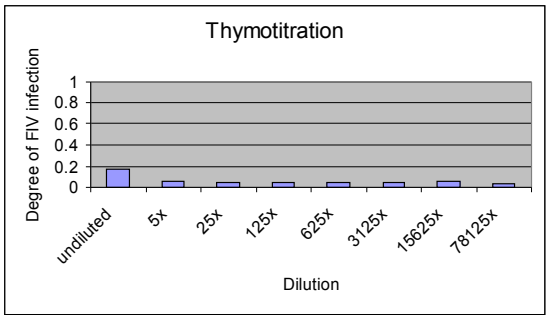
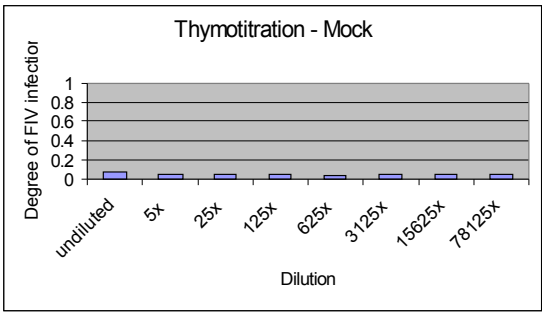


Figure 2: Thymotitration - Mock



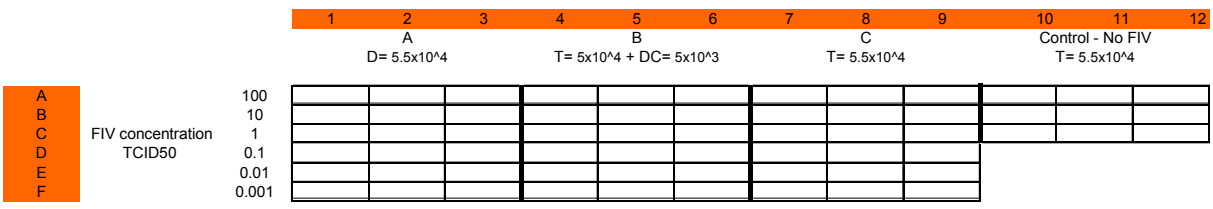
Dilution				
Undiluted	t Stat	3,101258	t Critical	2,776451
5x	t Stat	1,425343	t Critical	2,776451
25x	t Stat	-0,39268	t Critical	2,776451

It is however important to interpret these results with caution: results of earlier FIV infection experiments performed at the department of Virology and results from later experiments described here show considerable higher values up to 1.9 for the category 'undiluted'. It is possible that the ELISA was not performed in a correct way.

2.

A new category was added to the pilot experiment: thymocytes together with dc's. The ratio of dc's to thymocytes was 1:10.

Diagram 2: Setup Thymocytes + DC's +/- FIV-titration



The dc's are expected to present the antigen to thymocytes in an efficient way which should result in a higher infection with virus particles and the replicating of more virus. The procedure followed equals the procedure of the pilot experiment except for the addition of the dc's.

These were added in a concentration of $5x10^3$ in 50 µl of Iscoves' complete medium. As a negative control group 6 wells of the used 96 wells plate were filled with $5.5x10^4$ thymocytes in 50 µl of Iscoves' complete medium per well completed with 150 µl of Iscoves' complete medium. After six days of incubating at 37 °C 135µl of supernatant was transferred into a fresh 96 wells roundbottom plate. With this supernatant a p24 ELISA was performed. During this ELISA an unknown error occurred resulting in a fully negative plate including the added positive control. Possibly the first step in the protocol, the coating with monoclonal antibody 11C7C7C1, went wrong.
One day later this ELISA was performed again with the remaining supernatant, 65µl per well. This ELISA went successfully regarding the positive and negative results of the matching controls. However, the amount of antigen was only half the required amount; the results may therefore appear in a rather haphazardly fashion. The performed Students' T-test, Two-Sample Assuming Equal Variances showed –only just- no significant differences between the groups 'thymocytes with FIV' and 'thymoytes with FIV and DC's':

Figure 3: Thymocytes / FIV

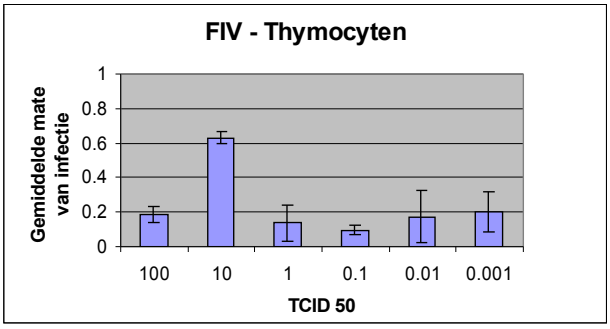
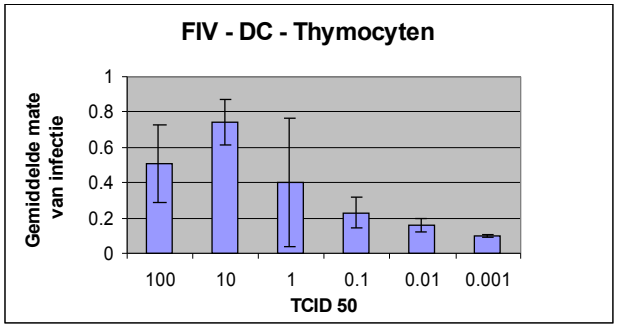


Figure 4: Thymocytes / FIV / DC's



T test FIV-DC-Thymocytes vs. FIV-Thymocytes

Dilution				
100	t Stat	2.461048963	t Critical two-tail	2.776451
10	t Stat	1.459495186	t Critical two-tail	2.776451
1	t Stat	1.230401749	t Critical two-tail	2.776451
0.1	t Stat	2.533637248	t Critical two-tail	2.776451
0.01	t Stat	0.167084327	t Critical two-tail	2.776451
0.001	t Stat	1.564320854	t Critical two-tail	2.776451

Yellow = significant (> 2,776451)
Red = not significant (< 2,776451)

3.
Because of the random results the experiment was repeated two weeks later following the same procedure, resulting as follows:

Figure 5: Thymocytes + FIV

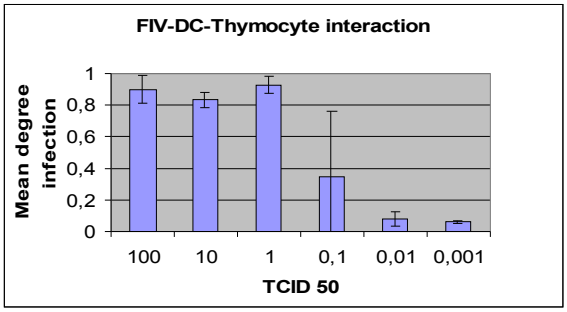
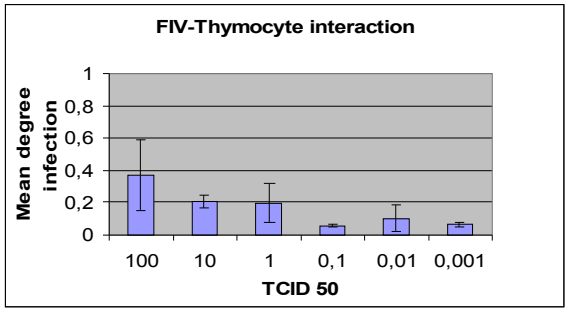


Figure 6: Thymocytes + FIV + DC's



T test FIV-DC-Thymocytes vs. FIV-Thymocytes

Dilution				
100	t Stat	3.877294124	t Critical	2.776451
10	t Stat	17.20692420	t Critical	2.776451
1	t Stat	9.469981059	t Critical	2.776451
0.1	t Stat	1.207396768	t Critical	2.776451
0.01	t Stat	0.389200445	t Critical	2.776451
0.001	t Stat	0.532152084	t Critical	2.776451

Yellow = significant (> 2,776450856)
Red = not significant (< 2,776450856)

4.
In order to examine the dc's need to mature in order to yield an effective viral presentation an adjustment to the former experiments was developed. Two categories of thymocytes to which dendritic cells were added were created. The first category used the same setup as experiments 2 and 3. In the second category the thymocytes were added after 24 hours. Thus, the dendritic cells could take up the virus and were able to mature for 24 hours. The setup:

Diagram 3: Setup FIV-DC-Thymocytes direct vs. FIV-Thymocytes

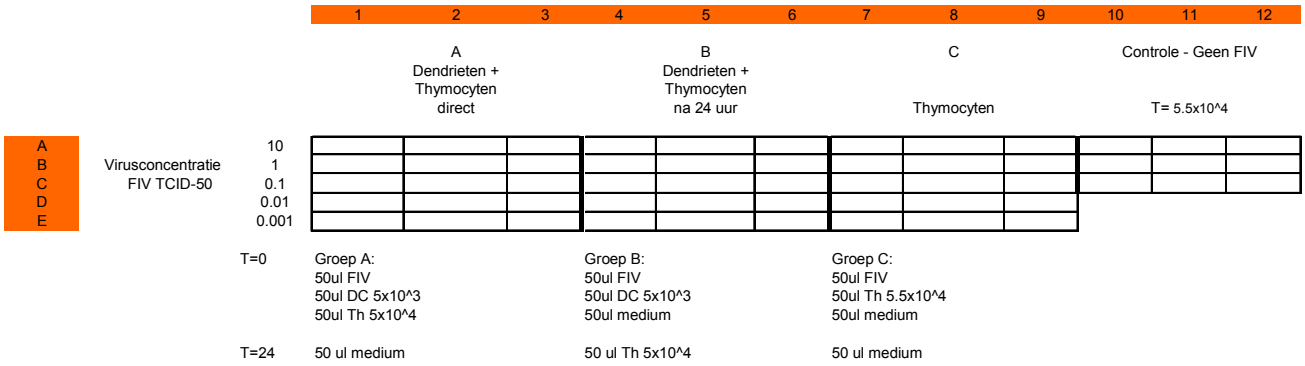


Figure 7: Thymocytes + DC's + FIV

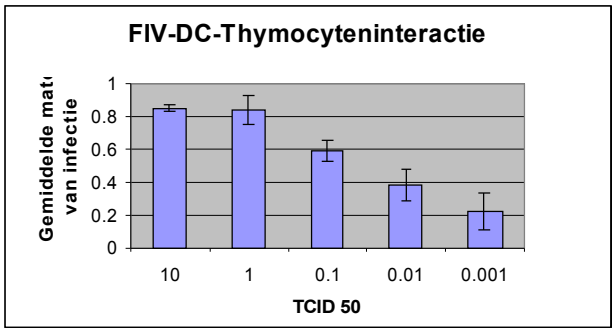


Figure 8: Thymocytes + DC's Delayed + FIV

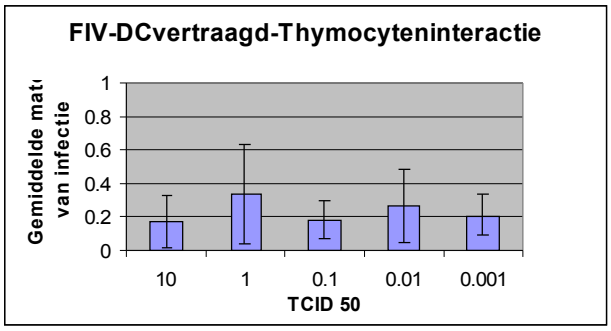


Figure 9: Thymocytes + FIV

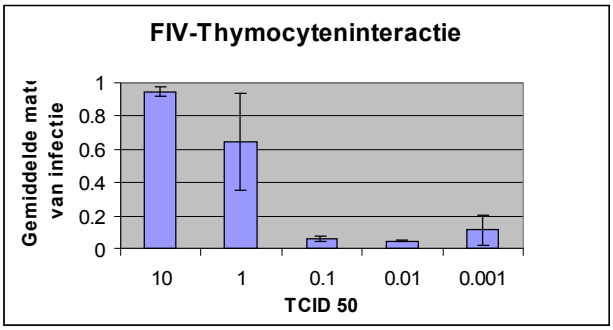
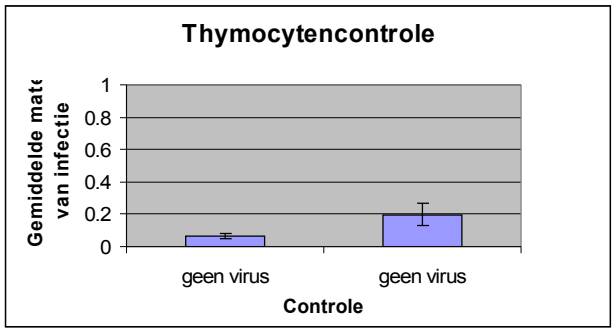


Figure 10: Thymocytes



T test FIV-DC-Thymocytes Direct vs. FIV - Thymocytes

Dilution				
10	t Stat	-4.52063	t Critical	2.776451
1	t Stat	1.143647	t Critical	2.776451
0.1	t Stat	13.60898	t Critical	2.776451
0.01	t Stat	6.103519	t Critical	2.776451
0.001	t Stat	1.319043	t Critical	2.776451

T test FIV-DC-Thymocytes Delayed vs. FIV-Thymocytes

Dilution				
10	t Stat	-8.33115	t Critical	2.776451
1	t Stat	-1.28256	t Critical	2.776451
0.1	t Stat	1.774843	t Critical	2.776451
0.01	t Stat	1.697348	t Critical	2.776451
0.001	t Stat	0.997671	t Critical	2.776451

Yellow = significant (> 2,776451)
Red = not significant (< 2,776451)

5.
After the former experiments in which thymocytic cells were infected with FIV, PBMC's were used. In the first experiment with these cells PBMC's together with DC's in a concentration of 10:1 with or without FIV were added to a well in a 6-well plate. The setup:

Diagram 4: PBMC's-DC's with FIV vs. PBMC's-DC's without FIV

Well →	1	2	3
	$3.75x10^6$ PBMC $3.75x10^5$ DC 100 $TCID_{50}$ Virus	$3.75x10^6$ PBMC $3.75x10^5$ DC 100 $TCID_{50}$ Virus	$3.75x10^6$ PBMC $3.75x10^5$ DC 100 $TCID_{50}$ Virus
Well →	4	5	6
	$3.75x10^6$ PBMC $3.75x10^5$ DC No Virus	$3.75x10^6$ PBMC $3.75x10^5$ DC No Virus	$3.75x10^6$ PBMC $3.75x10^5$ DC No Virus

The ELISA was performed after 7 days of incubation achieving the following results:

Figure 11: PBMC's + DC's + FIV

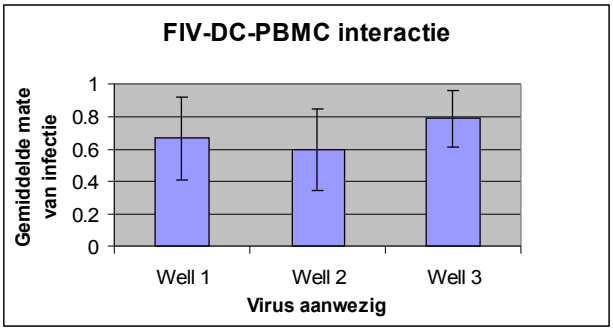
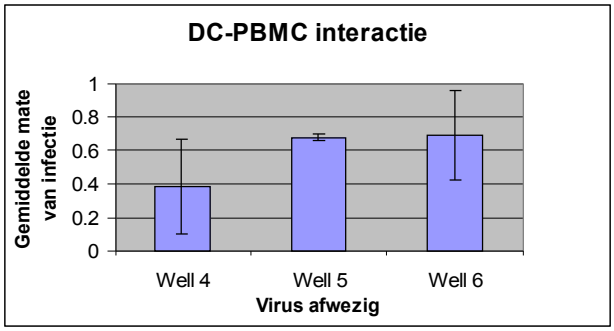


Figure 12: PBMC's + DC's



These results appear fully random with a large variance. The performed Students' T-test of the mean on the mean of the wells without FIV vs. the mean of the wells with FIV shows no significant result:

T test PBMC's-DC's with FIV vs. PBMC's-DC's without FIV

Dilution 10	t Stat	0.886917	t Critical	2.119905

The 6-well plate in which the PBMC's and DC's were incubated was re-examined following the ELISA. A large number of cell clusters was noticed in the wells. This is in accordance with the earlier mentioned strong adhesion of PBMC's after thawing and centrifuging and the slow resuspending of these clusters in fresh medium. When performing the ongoing experiments these findings were taken in account by letting the cells resuspend overnight in 100 mm Petri dishes after thawing and resuspending in order to prevent cell adhesion.

6.

After the first PBMC experiment the research question was extended: speed of transfer between PBMC's and DC's, the optimum concentration between PBMC's and DC's and the sensitivity of PBMC's for different concentrations of the virus were examined in the following setup:

Diagram 5: Setup of FIV-DC-Thymocytes vs. FIV-Thymocytes

			1	2	3	4	5	6	7	8	9
Four sets of plates											
				A			B			C	
Plate A			DC:PBMC	DC:PBMC	DC:PBMC	DC:PBMC	DC:PBMC	DC:PBMC	DC:PBMC	DC:PBMC	DC:PBMC
	A	0	1:10	1:10	1:10	1:100	1:100	1:100	1:1000	1:1000	1:1000
	B	100	1:10	1:10	1:10	1:100	1:100	1:100	1:1000	1:1000	1:1000
Dilution	C	10	1:10	1:10	1:10	1:100	1:100	1:100	1:1000	1:1000	1:1000
	D	1	1:10	1:10	1:10	1:100	1:100	1:100	1:1000	1:1000	1:1000
	E	0,1	1:10	1:10	1:10	1:100	1:100	1:100	1:1000	1:1000	1:1000
	F	0,01	1:10	1:10	1:10	1:100	1:100	1:100	1:1000	1:1000	1:1000
	G	0,001	1:10	1:10	1:10	1:100	1:100	1:100	1:1000	1:1000	1:1000
Plate B				D			E			F	
			PBMC	PBMC	PBMC	DC	DC	DC	Medium	Medium	Medium
	A	0	5*10^5	5*10^5	5*10^5	5*10^3	5*10^3	5*10^3	100 ul	100 ul	100 ul
	B	100	5*10^5	5*10^5	5*10^5	5*10^3	5*10^3	5*10^3	100 ul	100 ul	100 ul
Dilution	C	10	5*10^5	5*10^5	5*10^5	5*10^3	5*10^3	5*10^3	100 ul	100 ul	100 ul
	D	1	5*10^5	5*10^5	5*10^5	5*10^3	5*10^3	5*10^3	100 ul	100 ul	100 ul
	E	0,1	5*10^5	5*10^5	5*10^5	5*10^3	5*10^3	5*10^3	100 ul	100 ul	100 ul
	F	0,01	5*10^5	5*10^5	5*10^5	5*10^3	5*10^3	5*10^3	100 ul	100 ul	100 ul
	G	0,001	5*10^5	5*10^5	5*10^5	5*10^3	5*10^3	5*10^3	100 ul	100 ul	100 ul

Four equal plates of each category were incubated. Every three to four days samples were taken and stored in a 96 wells plate in the -20°C refrigerator. We planned to continue this procedure for two weeks in order

to determine the date on which the PBMC's appear FIV positive in the p24 ELISA. After two days yeast overgrowth appeared in some of the category A and B wells on the A-plates. These wells were emptied; nevertheless samples were taken from these plates. Two days later yeasts turned up again in the above mentioned categories on the A plates. These wells were also emptied. The last A plate to be sampled turned out to be almost fully overgrown by yeasts resulting in the removal of this plate from the experiment.
An other setback was the tipping of two of the B plates in the incubator. These plates also had to be removed from the experiment.
Eventually three A plates and two B plates remained to be sampled and examined in the p24 ELISA. The results from these plates were disappointing: positive results appeared randomly dispersed among all the FIV-dilutions of DC and PBMC combinations. It was not possible to conclude anything from this result. Positive and negative controls yielded their respective outcome.
It was however possible to indicate a time trend from which positive results emerge: in the first plate sampled after five days only a few lightly coloured and thus slightly positive wells show up whereas the second batch of samples (at day 9) show positive wells, although they are distributed in a random way. The results:

Figure 13: PBMC's + DC's + FIV 1:10

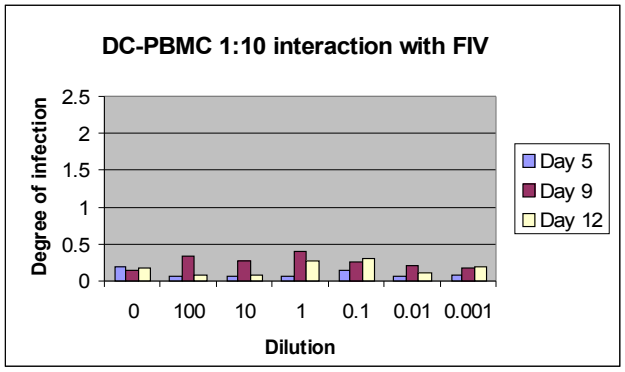


Figure 14: PBMC's +DC's + FIV 1:100

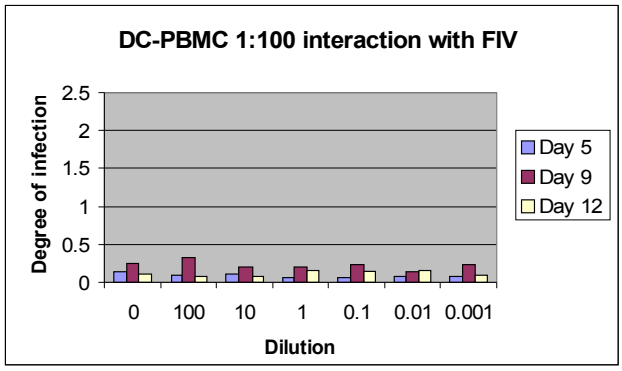
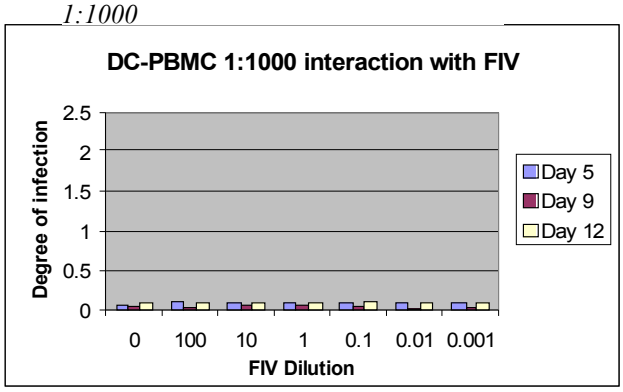


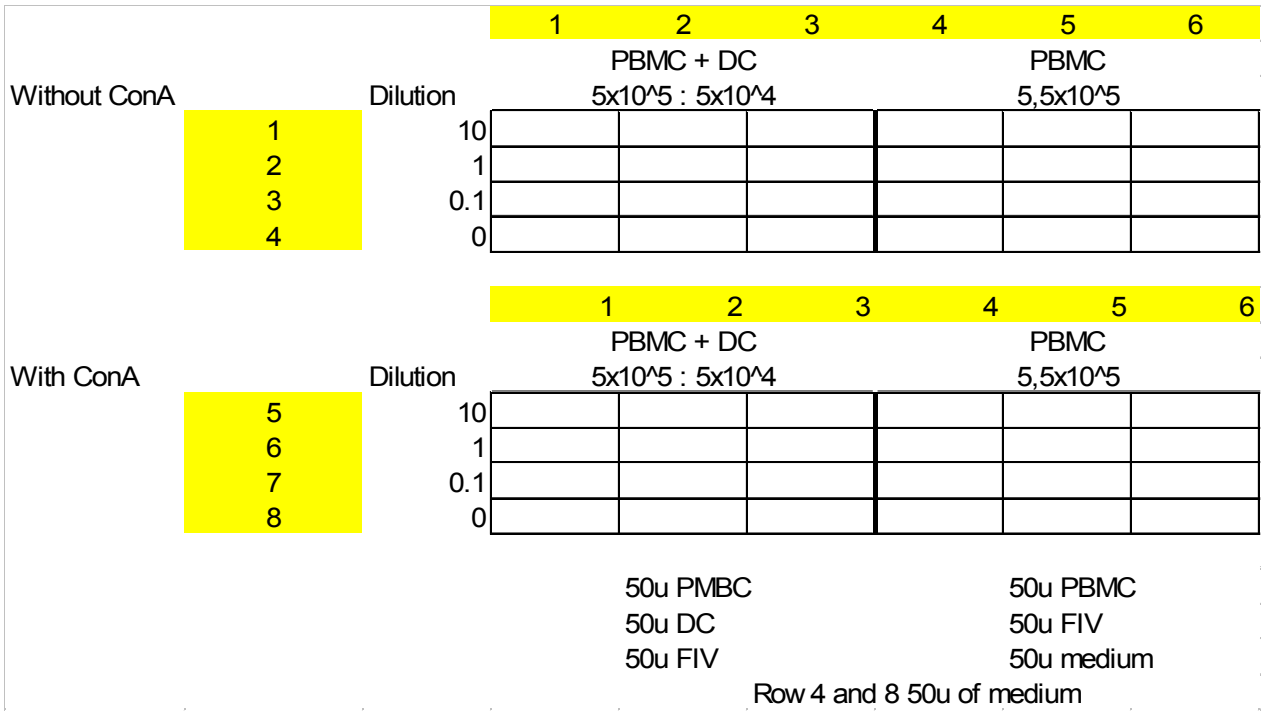
Figure 15: PBMC's + DC's + FIV 1:1000



7.

During the course of the above described experiment a similar experiment was set up in which PBMC's were cultured in two ways after thawing: one category consisted of PBMC's being cultured in 'conventional' IL-2 containing medium whereas the other category PBMC's were cultured in Concanavalin A containing medium. To both categories three different concentrations of FIV and one negative control consisting of only medium were added. To half the PBMC containing wells DC's were added in a concentration of 10:1. The setup:

Diagram 6: Setup of PBMC's-DC's without ConA vs. with ConA



Contrary to the former experiment samples were only taken on day seven. This resulted in the following:

Figure 16: PBMC's + DC's + FIV + ConA

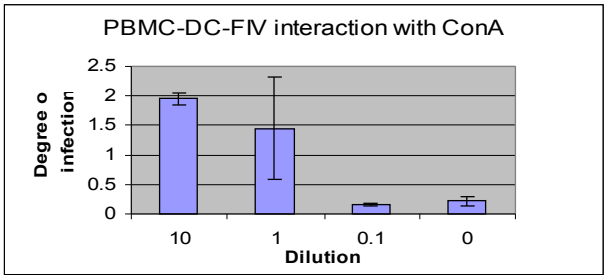


Figure 17: PBMC's +DC's + FIV

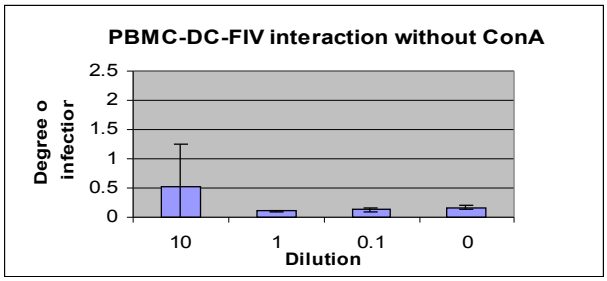


Figure 18: PBMC's + FIV + ConA

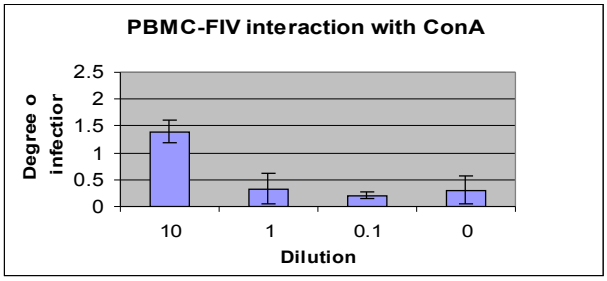
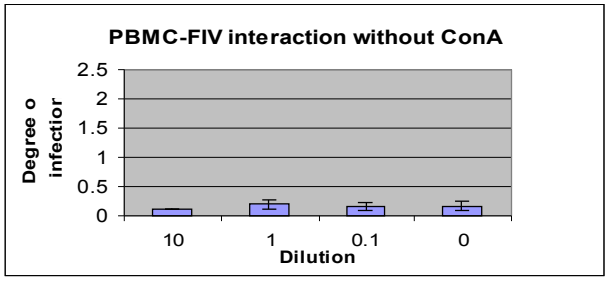


Figure 19: PBMC's + FIV



T-test PBMC's-DC's with ConA vs. PBMC's-DC's without ConA

Dilution				
10	t Stat	-3.38331	t Critical	2,776451
1	t Stat	-2.65529	t Critical	2,776451
0.1	t Stat	-1.07089	t Critical	2,776451
0	t Stat	-0.99136	t Critical	2,776451

T-test PBMC's with ConA vs. PBMC's without ConA

Dilution				
10	t Stat	-11.0125	t Critical	2,776451
1	t Stat	-0.8	t Critical	2,776451
0.1	t Stat	-0.76209	t Critical	2,776451
0	t Stat	-0.8499	t Critical	2,776451

Yellow = significant (> 2,776451)
Red = not significant (< 2,776451)

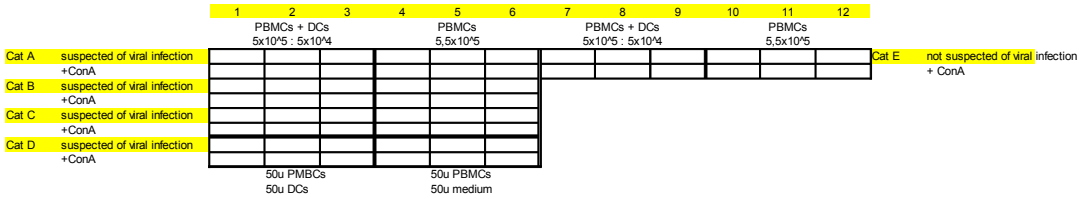
Regarding the higher yield of positive results in the 'Dilution 10' category ConA is expected to improve the infection rate of the PBMC's. ConA is therefore also used in the following experiments.

8.

In the next series of experiments known positive and negative cat PBMC's were used in combination with DC's against a control set of PBMC's without DC's. Cat PBMC's used were cat 80, cat 308, cat 336, cat 'Babette' and cat 'Jacob'. The cells were cultured in standing T_{25} containers in order

to minimize possible yeast or funghal infections because of the small opening of these containers. Cells were stimulated for two days with ConA after the addition of the DC's. Setup:

Diagram 7: Setup known FIV cats – PBMC's with or without DC interaction and with or without ConA interaction

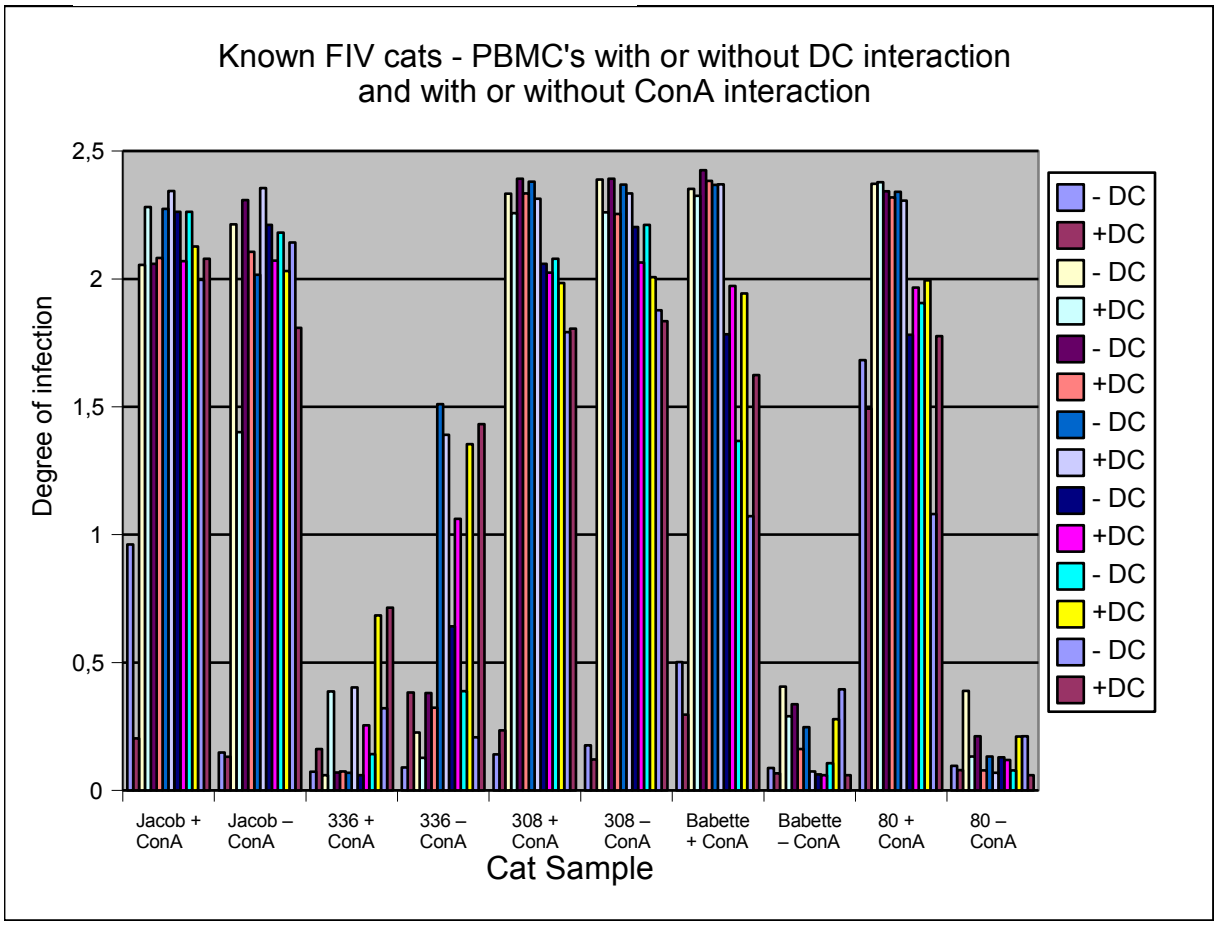


		PBMCs + DCs 5x10^5 : 5x10^4	PBMCs 5,5x10^5
Cat A	suspected of viral infection	T25	T25
	+ConA	T25	T25
Cat B	suspected of viral infection	T25	T25
	+ConA	T25	T25
Cat C	suspected of viral infection	T25	T25
	+ConA	T25	T25
Cat D	suspected of viral infection	T25	T25
	+ConA	T25	T25
Cat E	not suspected of viral infection	T25	T25
	+ConA	T25	T25

After 3 to 4 days samples are taken from each T25, put into a 96 wells plate and refridgerated at -20 degrees Celsius
In total 20 T25s are needed
All PBMCs and thymocytes are stimulated by means of ConA

Samples were taken and frozen at a 3-4 days interval scheme during a period of three weeks to examine the date(s) on which the p24 ELISA yielded a positive result. The medium was refilled whenever the samples were taken. After three days a lot of cellular debris was observed in the T_{25} containers. The results:

Figure 20: Known FIV PBMC's +/- DC's, +/- ConA

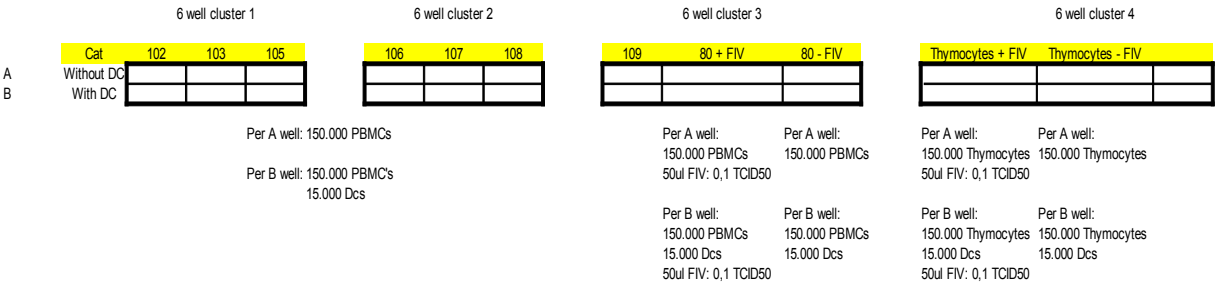


Conclusion: Concanavalin A seems to have a large effect on Babette and Cat 80 samples, which is not to be expected. It should propagate the infection of the PBMC's with FIV if the virus is present, but not to this extent. The other major problem lies with the apparent yield of a positive result of the Cat 80 PBMC's which should not be infected with FIV, Cat 80 being the negative control cat regarding FIV.
DC's do not show a consistent propagation of FIV infection regarding the miscellaneous positive and negative effects they present in the above graph.
The only effect which can be detected from the experiment again seems to be the time effect in which infections take place between three and seven days.

9.

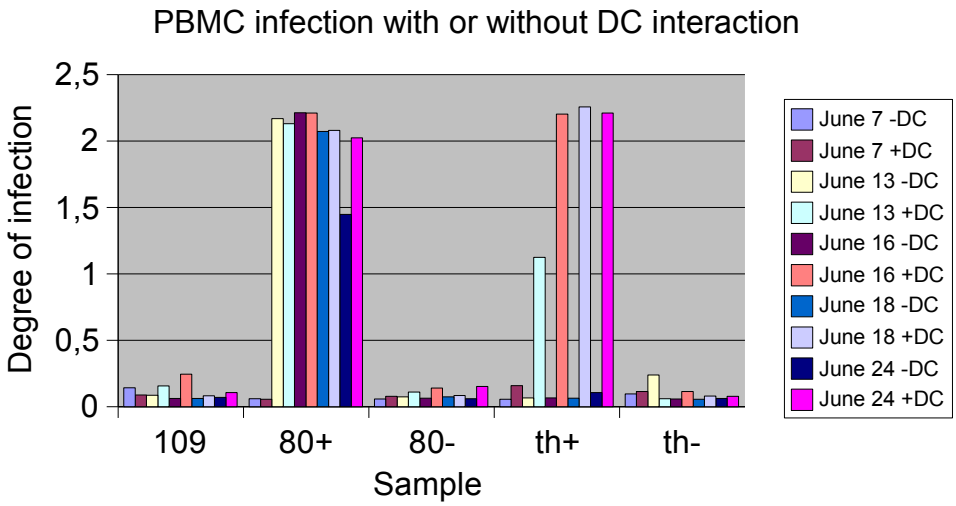
In this experiment sets of positive and negative cat PBMC's (cat 102, cat 103, cat 105, cat 106, cat 107, cat 108 and cat 109) together with DC's and together with control sets of cat 80 PBMC's with or without FIV and thymocytic cells with or without FIV, with or without DC's were cultured during a period of three weeks. These cultures were -contrary to the former experiment- performed in 6 well plates. All cultures were stimulated with Concanavalin A for two days. Every three or for days samples were taken from all the wells which were subsequently frozen at -20° C. The medium level was readjusted whenever the samples were taken. After three days very few PBMC's were observed using the light microscope in the 102 to 109 series cat PBMC cultures. After six days all these PBMC's showed cyto pathic effect (cpe). The reason of the cpe is unknown; an explanation could possibly lie in bacterial growth or an originally bad refrigeration process. The DC's in these wells performed well as did the cat 80 PBMC's and the thymocytic cells. These well performing PBMC's were cultured subsequently as a repetition of earlier experiments. The original setup:

Diagram 8: setup PBMC's with unknown infection with/without DC's



The results show a confirmation of the former experiments: the FIV infected cat 80 PBMC's and the FIV infected thymocytic cells show a positive result after 9 days but not after three days. There is no clear effect from the DC's in the cat 80 PBMC's when the +DC and –DC categories are compared regarding the positive outcome after 9 days. Strangely the –DC category FIV positive thymocytic cells were not positive tested at all. An explanation could be a failure of adding FIV to these category of thymocytic cells.

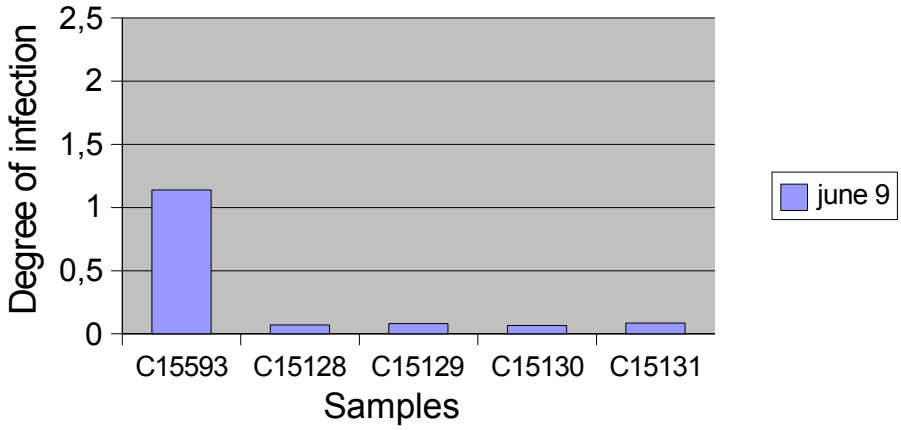
Figure 21: PBMC's +/- DC's, +/- FIV + ConA



10.

As the final experiment 5 samples (C15593, C15128, C15129, C15130 and C15131) containing PBMC's from field cats whose FIV status was thusfar unknown were thawed. These cells were cultivated for three days in standing T_{25} containers filled with Iscoves' complete medium containing Con A which was replaced with IL-2 containing Iscoves' complete medium on day four. This growing sequence was repeated the second week to gain a large batch of cells of which part could be refrigerated again for future research. A baseline measurement of FIV infection was taken after one week in culture:

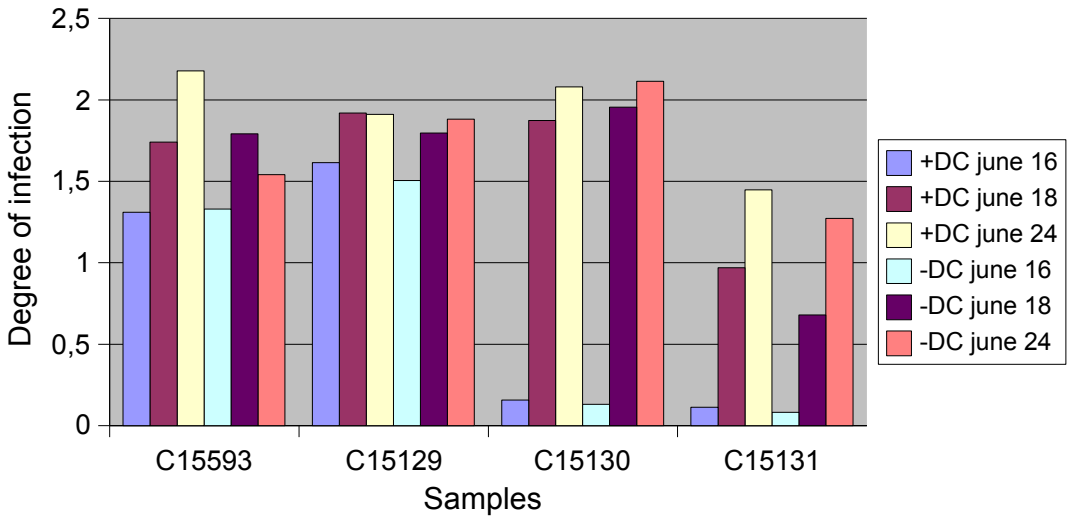
Figure 22: Baseline measurement of FIV infection



As seen above, sample C15593 already shows a positive FIV result before any addition of cells at day 5 (day 0 was June 4th).
After two weeks of culturing cells from sample C15129 did not replicate anymore. Very few PBMC's resided in the container compared to the other containers. Furthermore they seemed to be degenerated when evaluated using the light microscope. This sample was excluded from the

experiment. At day 10 the DC's were added in a concentration of 1:10 compared to the PBMC's. Samples were taken after 12, 14 and 20 days (counting from day 0). The following results were obtained:

Figure 23: Field cat PBMC infection with or without DC interaction



As can be seen above all samples were positive although sample C15131 only became positive after 20 days. DC's do not seem to have any influence on the process.

Discussion

The first range of experiments showed that thymocytic cells are infectable with FIV. Furthermore, dc's appear to shorten the timeframe in which FIV is for the first time replicated by thymocytes. This potentially decreases the time necessary to yield positive ELISA test results. On the other hand, premature dendritic cells does not appear to have an effect on the ELISA results obtained.

The results were difficult to interpret in the experiments using PBMC's in contrast to the results obtained when thymocytic cells were used. In only one of the experiments performed with PBMC's and dendritic cells the dc's seemed to increase the amount of FIV reproduced by the PBMC's. In the same experimental setup stimulation of the PBMC's by Concanavalin A appeared to increase the amount of FIV reproduced. Therefore subsequent experiments were performed using Concanavalin A as a stimulant in the culturing process. The role of Concanavalin A might be an interesting future research goal.

Regarding PBMC's derived from FIV infected patients the effect of dendritic cells was questionable. Most PBMC-DC interactions showed no effect at all regarding the moment of FIV replication or only random results were obtained. This might be a consequence of very rapid develoment of positive p24 ELISA results by most PBMC's from FIV infected patients in both the control group and the DC challenged group.

It might be possible that the matching of PBMC's and DC's is host specific e.g. Cat A PBMC FIV production is only effectively influenced by Cat A DC's and not Cat B DC's, by lacking certain host specific adhesion molecules on their outer cell surface.

Regarding the results of all the performed experiments it is important to mention the weak statistical base of the above conclusions: most of the statistical results are based on only three samples.

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I would especially like to thank Nancy, Arno and Eddy who assisted me in many ways during my first weeks in the virology labs and later on. Many thanks go to Frank van der Meer for his daily guidance ... and patience. Finally I would like to thank Herman Egberink for offering the opportunity to perform this research project at the department of Virology and for his surveillance of the project at a small distance.

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