The role of the Dendritic Cell in the Immunobiology of Feline Infectious Peritonitis

Research internship

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ABSTRACT

Feline Infectious Peritonitis (FIP) is a fatal disease in cats caused by a Coronavirus. During FIPv infection the T-cell population is depleted. This is seen in many other diseases, such as measles and AIDS. In these two diseases, the Dendritic Cell (DC) has been shown to play a role in T-cell depletion. This study endeavours to prove if such a mechanism exists in FIP.

In order to investigate permissiveness of DCs for FIPv, a growth curve was produced. This growth curve showed prolonged viral reproduction to intermediate titres. As T-cell depletion during FIPv infection occurs at least partly through apoptosis, a TUNEL assay was used to evaluate this. FACS analysis of infected and non-infected co-cultures of DCs with Thymocytes revealed no difference in apoptosis levels. These data suggest that T-cell depletion during FIPv infection does not occur through apoptosis initiated by FIPv-infected DCs.

INTRODUCTION

Feline Infectious Peritonitis (FIP) is not a common disease in cats, but it is usually fatal. Its clinical signs are not specific and laboratory tests do not yield specific results, therefore making a definite diagnosis of FIP is difficult.[13] Moreover, the effectiveness of the available vaccine is not universally agreed upon[6, 16]. These characteristics render FIP a complex disease, and any research clarifying prevention, pathogenesis, diagnosis or treatment is welcomed.

aetiology

FIP is caused by a virulent mutant of the common Feline Enteric Coronavirus (FECV).[6, 16] The genus Coronavirus, member of the family Coronaviridae of the order Nidovirales, contains three groups of viruses. The feline coronaviruses (FCoV) can be found in group I, together with Canine Coronavirus (CCV) and Transmissible Gastroenteritis Virus of swine (TGEV).[15, 21, 24]

Coronaviruses are positive-stranded RNA viruses. RNA polymerase has no proofreading functionality, resulting in a high mutation rate.[16] Moreover, coronaviruses replicate their RNA in a discontinuous manner through polymerase jumping, leading to even more mutation. [18] The consequence is that even in the same host the virions do not contain exactly the same genetic material. The group of semi-alike viruses within one host is referred to as *quasispecies*.[7, 16]

During infection with FECV, there is a possibility that the quasispecies gives rise to a virulent mutant. If this mutant causes a syndrome with the pathogenic characteristics of FIP, the mutant is designated as FIPv. The longer a FECV infection persists, the higher the chance that such a mutation arises. Once mutated, the FIP virus loses much, if not all, of its infectivity. In this way every patient will culture its own version of the virus.[16]

There are currently two serotypes of FIPv, type I which grows poorly in cell culture, and type II which is related to CCV. As type II more readily grows in cell culture it has been most extensively studied, even though type I is more prevalent in the field.[6, 15, 16, 21]

epidemiology

FECV is common in cat populations, especially in crowded conditions such as catteries and animal shelters. Many cats encounter FECV at some time during their life. FECV infection in itself does not cause dangerous disease, and most cats will eliminate the virus within a few weeks.

In some cats, however, the immune system is not able to eliminate the virus and the infection will persist. In the continued replication during persistent infection there is a chance that mutation will occur. If a virulent mutant emerges, it will cause the systemic infection known as FIP.

pathogenesis

The pathogenesis of FIP is complex and not yet completely understood. The original virus, FECV, infects the bowel, where it causes mild gastrointestinal symptoms or no symptoms at all. FECV remains mainly in the digestive tract, though some virus may be found systemically.[21]

If FECV mutates into the more virulent phenotype that induces FIP, a new pathologic pathway emerges. In the initial stages, macrophages will be infected leading to a more generalised infection.[5, 6, 16]

The second most important step is the modulation of the immune response towards an inappropriate humoral response.[5] The B-

cells are induced to produce an excessive amount of antibodies directed against the virus.
These antibodies cause most of the pathology because they do not neutralise the virus. Instead, when they bind to free virus they form immune complexes (ICX). ICX are clumps of virus particles with several antibodies attached. These ICX run aground in capillaries in many parts of the body. The antibodies contained in the ICX carry signal sequences that induce the immune system to start a local inflammatory response. Although this is generally useful in clearing pathogens, in this particular case it is the main source of symptoms.[6, 16]
While the B-cells are producing antibodies, the T-cells disappear.[5] This causes a reduced protection against other pathogens. Haagmans et al. have shown that the T-cells in a cat infected with FIPv undergo apoptosis, but the mechanism that induces the T-cells to take this drastic pathway is not yet clear.[13]

clinical presentation

The initial clinical signs of FIP are non-specific and include chronic intermittent fever, anorexia, weight loss, and lethargy[6, 16]. Later some more specific signs may occur, caused by the specific location in which the ICX have run aground. Examples are ocular lesions, and neurological signs.[6]
Two distinct syndromes of FIP have been described, though many cats display a hybrid of these. One syndrome is effusive or 'wet' FIP, in which the accumulation of fluid in abdomen, thorax, or pericardium is most pronounced. The other syndrome is proliferative or 'dry' FIP, in which granulomatous lesions are characteristic.[6] These lesions are often found in kidneys and brain, but no tissues are exempt. Of the two syndromes, wet FIP usually has the most rapid progress towards death.
The extent of immunosuppression may be the determining factor in which syndrome the patient will display. Wet FIP is assumed to occur with an immune response strongly skewed towards humoral immunity. It appears that when there is some cellular immunity left, the animal will more likely develop dry FIP.[13]

risk factors

There are several known risk factors for FIP. Cats younger than four years and those older than fourteen are more often diagnosed with FIP. Factors that have an immunosuppressive effect catalyse the development of FIP.[6, 16]
Immunosuppressive factors include stress and concurrent disease, most notably FIV and FeLV infections.[13] Another possible immunosuppressive factor is loss of genetic diversity, as can occur in pedigree cats. A likely example of this phenomenon is the Cheetah, a species known to have low genetic diversity that appears to be exceptionally vulnerable to FIP.[6, 14] In multiple-cat facilities such as catteries and shelters, cats not only have to cope with an increased chance of becoming infected with FECV, but also with the stress these situations cause to the preferentially solitary domestic cat.

diagnosis

The suspicion that a patient suffers from FIP can be further strengthened by haematology, serum chemistry, serology findings, and examination of the ascitic fluid. The abnormalities shown in table 1 are not always seen in a cat with FIP, and can also occur in cats with other diseases, but if more of these abnormalities occur in the same patient the suspicion of FIP increases.
Although some clinicians like to call the FECV titre a 'FIP titre' it is important to note that any cat that has encountered FECV might have antibodies against Feline Coronavirus. The presence of these antibodies is not proof of FIP. The diagnosis can only be confirmed by pathological examination.[16]

Type of examination	Abnormality
Haematology	leukocytosis with neutrophilia and lymphopenia
	anaemia
Serum chemistry	decreased albumin/globulin ratio
	hypoalbumineamia
	hyperglobulinaemia
	coagulopathy
	raised urea and creatinin values
	hyperbilirubinaemia
	elevation of enzyme levels
Serology	high titre for FECV
Ascitic fluid	yellow
	viscous
	coagulates when exposed to air
	high amount of γ-globulins
	high number of cells: neutrophils, lymphocytes, macrophages, mesothelial cells

Table 1: Abnormalities often found in cats with FIP[13, 16]

prevention

No undisputedly effective vaccine has been identified yet; moreover, many vaccines exacerbate the course of the disease. If a vaccine induces humoral immunity, there is a high chance that antibody dependent enhanced disease occurs. When this happens, the animal will succumb to FIP much faster than it would have had it not been vaccinated.[5, 6, 16, 21]

Another strategy for prevention of FIP is prevention of FECV infection. Because FECV is endemic in many catteries, this would entail separating kittens from the queen directly after birth and raising them by hand. This is stressful for both kittens and queen, and deprives the kittens of colostrum. Furthermore, the efficacy of this strategy has not yet been proven. Therefore, this is not a method to be used indiscriminately.[16]

T-cell depletion

The lymphopenia found in FIP patients is mainly caused by depletion of the T-cell fraction. This phenomenon is at least partially due to apoptosis of T-cells in the lymphatic tissues. Haagmans et al. found evidence of apoptotic T-cells in tissues taken during post-mortem examination of laboratory cats that had succumbed to FIP.[13] Furthermore, it was found that T-cells from a FIPv-infected cat, when stimulated, do not undergo mitosis but instead enter an apoptotic pathway. T-cells obtained from healthy cats could be induced to undergo apoptosis through contact with ascitic fluid of a FIPv-infected cat.[13]

possible mechanisms of T-cell death

Feline Infectious Peritonitis is not unique in targeting host T-cells. Many other pathogens do the same, for instance Measles Virus, and Human Immunodeficiency Virus (HIV).[1, 10, 12, 22, 23, 25]

In these cases the Dendritic Cell (DC) has been implicated as the catalyst through which the pathogen performs its immuno-suppressive action. This is not wholly surprising, as the DC is the primary antigen presenting cell of the immune system and is regularly in close contact with T-cells. The DCs have an intricate interaction with the T-cells, playing an important role in their activation and survival.[1, 2, 10-12, 17, 20, 22, 23, 25]

There are many different ways in which a pathogen can influence or abuse the DC–T-cell interaction. HIV -and probably the related FIV as well- binds a surface molecule specific to DCs, DC-SIGN. The virus is then internalised, but somehow manages to prevent being processed and presented on the MHC. When the DC initiates interaction with a T-cell, the HIV virus uses this interaction to be delivered to the T-cell, which it subsequently infects.[9, 11, 25]

In measles virus infection, the most important difference is that the T-cell is not infected. Measles virus infects DCs and alters their expression of interleukins, thus altering their effect on the T-cell. As the T-cells die by another mechanism than direct virus infection, this is classified as a *bystander effect*.[1, 10, 12, 22, 23]

If in FIP the apoptosis is indeed catalysed by DCs, the mechanism is most likely one such as described for measles, for none of the apoptotic T-cells were found to be infected themselves.[13]

This study focuses on the role of the Dendritic Cell in the occurrence of T-cell apoptosis in cats infected with FIPv. Because experimentally infecting cats with a potentially fatal virus raises ethical concerns that should not be taken lightly, it is valid to start with a study of the phenomenon *in vitro*.

To this purpose, a co-culture of T-cells with DCs infected with FIPv is studied. Instead of T-cells, Thymocytes are used as they are accepted stand-ins for T-cells in *in vitro* studies. Dendritic cells are cultured *in vitro* from myeloid progenitor cells.

MATERIALS AND METHODS

culturing of cells

In the course of this study several different cell types were needed: FCWF cells, bone marrow derived Dendritic Cells, and Thymocytes. The following section describes the methods of producing and maintaining these cells.

FCWF cells

Felis Catus Whole Fetus (FCWF) cells are widely used in research on feline

coronaviruses because they are highly susceptible to FIPv.[4-6]
FCWF cells are cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 100 IU/ml penicillin, and 10 µg/ml streptomycin. They are kept at 37°C and an atmosphere with 5% CO_2. Every two to three days the cells are passaged to another container to make sure there is never more than a monolayer of cells and to stimulate growth. Passaging the cells occurs in four major steps: first the cells are washed with Phosphate Buffered Saline (PBS0). After washing, the cells are treated with Trypsin to digest the connections between the cells and the flask. When the cells have detached from the flask and from each other they are resuspended in medium. The last step before the cells are moved into the new flask is to dilute the cells to an appropriate concentration. In FCWF cells the appropriate dilution is usually somewhere between one in two and one in five.
The number of passages is recorded and when the number approaches eighty it is usually time to switch to a fresh batch of FCWF cells. This is necessary because older lines of FCWF cells start to change their properties. For instance, they become more attached to their surroundings and have a slower growth rate.

Bone marrow derived cells

Bone marrow contains myeloid progenitor cells, which can give rise to both Macrophages and Dendritic Cells (DCs). Feline DCs are cultured from feline bone marrow. For every test a new batch has to be cultured, because the cells do not have a long life in culture. This poses a problem in ensuring that the cells are sufficiently comparable. Therefore, before starting the tests, bone marrow from one laboratory cat was frozen in quantities sufficient for the planned tests.

freezing bone marrow

When a cell is frozen, the water in the cell forms crystals that may damage the cell. To circumvent this problem, cells can be frozen in Dimethylsulfoxide (DMSO). DMSO replaces the water in the cell so that the cell can be safely frozen. However, using DMSO creates a new problem: at temperatures above 20°C DMSO is toxic to the cell. Therefore cells need to be cooled whenever they are in contact with DMSO. Furthermore, it is important to prevent sudden changes in temperature or in DMSO concentration as these will also harm the cells.
After the bone marrow is harvested from the animal it is brought into a suspension of $3.75*10^6$ to $7.5*10^6$ cells/ml. The suspension is then cooled and kept on ice throughout the following procedure. A cold solution of 20% DMSO in FBS is added drop by drop until the original volume is doubled. Thus, the resulting concentration of DMSO is 10%. The suspension is then distributed over the containers and frozen at –80°C. After they have been at –80°C for at least three days the containers can be moved to –150°C.

thawing bone marrow

When thawing cells, slowly removing the DMSO is most important. The suspension is allowed to warm just enough to become fluid, and then kept on ice. The DMSO is diluted by slowly adding cold DMEM. Once the DMSO has been diluted to approximately 1% the medium is removed by centrifuging. The remaining cell pellet is gently resuspended in warm culturing medium.

Dendritic Cells

After thawing the required progenitor cells there are two paths that can be taken towards producing Dendritic Cells: the conventional culturing method, or culturing of cells that express CD14. In both these methods the medium used is Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% FBS, 100 IU/ml penicillin, 10 µg/ml streptomycin, 0.05 mM β-mercapto-ethanole, 10 ng/ml Granulocyte Macrophage – Colony Stimulating Factor, and 10 ng/ml IL-4.[2, 10, 19, 24]

conventional culturing

The newly thawed cells are cultured for 24 hours in one well of a six-well cluster. Non-adherent cells are removed and are further incubated in the next well. The non-adherent cells are left to incubate another 24 hours, after which the procedure is repeated. The adherent cells are provided with fresh medium and every two days the medium is replenished. Following six to ten days of culturing the now non-adherent Dendritic Cells are harvested from the first two wells.[2, 19]

selection for CD14 using a Magnetism-Activated Cell Sorter (MACS)[20]

The progenitor cells for Dendritic Cells all express CD14. This feature can be used to select only Dendritic Cell precursors from bone marrow cells.
First, the recently thawed cells are washed with MACS buffer (PBS0 with 2mM EDTA and 2% FBS) and then incubated for 30 minutes at 4°C with α-CD14 immunoglobulins labelled with paramagnetic beads. After incubation, the cells are washed and resuspended in 0.5 ml MACS buffer. A column of iron mesh is placed within a strong magnetic field, moistened with 0.5 ml MACS buffer and then the cell suspension is allowed to pass through. Labelled cells will stick to the mesh while unlabeled cells pass, thus separating the two cell fractions. The column is washed thrice with 0.5 ml MACS buffer before it is removed from the magnetic field and rinsed to dislodge the labelled cells. The purified fraction can now be cultured without the need to remove non-adherent cells. Every two days fresh medium is supplied and after six to ten days of culturing the Dendritic Cells are harvested.

Thymocytes

Thymus cells taken from SPF cats are cultured in IMDM supplemented with 10% FBS, 100 IU/ml penicillin, 10 µg/ml streptomycin, 0.05 mM β-mercapto-ethanol, and 200 IU/ml IL-2. Every seven to ten days 5 µg/ml Concanavalin A is added to stimulate the cells to undergo mitosis. After 48 hours, the cells are washed, counted, and diluted to a concentration of $1*10^6$ cells/ml. When there has been an accumulation of dead cells in the Thymocyte suspension the cells can be cleansed by Ficoll-Hypaque™ density centrifugation. This procedure requires that the cells are suspended in PBS with 2mM EDTA and carefully pipetted onto the Ficoll-Hypaque™ mixture. The mixture is centrifuged, and the denser dead cells will pass through the Ficoll-Hypaque™, while the viable Thymocytes remain at the border between the two liquids.

the virus

Of the two serotypes of FIPv, type II will most readily grow in cell culture.[6, 21] It has therefore been most widely studied.[21] In line with previous research a stock was used of a type II FIPv: strain 79-1146.[4, 5] The virus content of this stock is $4*10^7$ $TCID_{50}$ per ml.

determining the virus content of stock and samples

For determining the virus content of samples and stocks the Tissue Culture Infective Dose 50% ($TCID_{50}$) test is used. The principle of this test is to make dilutions of the sample and incubate these with appropriate cells. After a while – different per cell type and virus – the cells are examined to detect cytopathogenic effect (CPE). From the number of wells affected per titration a titre can be calculated that is an indication of the amount of infective particles present in that sample.
When working with FIPv the cell type of choice is the FCWF cell. These cells are easily infected by FIPv and the CPE is easy to recognise, even without using any colouring technique. The cells are pipetted into microtitre plates with flat-bottomed wells at a concentration of $3*10^4$ cells per well and left to incubate overnight. The next day the wells are covered with a monolayer of FCWF cells that is 80% confluent. The sample is diluted in medium with 2% FBS and of each dilution 100 µl is added to at least three wells. The lower percentage of FBS serves to slow the growth rate of the FWCF cells. After an incubation period of five days the cells are examined for CPE.

calculating $TCID_{50}$values

Scoring for CPE generates raw data that must be processed to generate a value for the sample. The formula is given below:

$$TCID_{50} = 1/v * 10^{t - d/2 + d*\Sigma (r/n)}$$

Where v = volume, usually in ml; t = log (highest dilution where all wells showed CPE); d = log (dilution factor used); r = number of wells positive for CPE, upwards from and including the highest dilution where all wells were positive; and n = number of wells that were used per dilution.

counting cells

In several instances cells need to be counted. There are many ways to count cells and all have both advantages and disadvantages. The method most commonly used in our laboratory consists of mixing a small volume of cell suspension with the same volume of Trypan Blue. Trypan Blue enters only dead cells, as it cannot enter a cell with an intact membrane. The cells are left for about five

minutes and then counted using a microscope and a standard cell-counting slide. Viable cells appear pale in comparison to the blue background while dead or damaged cells are dark blue. The dye needs a few minutes to fully enter all damaged cells, but it should not be left for more than thirty minutes as the dye is toxic and this will lead to an overestimate of the number of dead cells in the culture. In calculating the cell concentration of the suspension it is important to realise what volume of the *original* suspension has been counted.

production of virus-stock

FCWF cells are ideal for producing FIPv stocks as they quickly produce virus to high titres. Inoculum containing 0.05 $TCID_{50}$ virus per cell is added to an FCWF culture that is 70% to 80% confluent. The culture is inoculated at the end of the day and the stock is harvested first thing in the morning, thus an incubation period of about 15 hours is accomplished. The medium is harvested and centrifuged for 10 minutes at 3000 rpm to get rid of cells and cell debris. The newly acquired stock is frozen at –80˚C and a sample is taken to determine the $TCID_{50}$ of this stock.

determining an appropriate multiplicity of infection

In the next experiments Dendritic Cells are needed that are infected with FIPv. As working with infected Dendritic Cells is a new avenue in this research, it was first necessary to find a multiplicity of infection (MOI) that yields adequate amounts of infected Dendritic Cells. To this end Dendritic Cells are incubated for 8 hours with different MOIs, ranging from 0.1 $TCID_{50}$ per cell to 500 $TCID_{50}$ per cell. FCWF cells are used as positive control. Following incubation, the cells are washed and prepared for either immunofluorescence staining or immunoperoxidase staining. If the staining is to be done on glass slides, the cells are resuspended in 40µl PBS0 per well, pipetted onto the slides and left to dry. This amounts to 7000 cells per well for DCs, and 5000 for FCWF cells. The cells are fixed onto the glass slides with a mixture of 5% acetic acid in methanol.

immunofluorescence staining (IFT)[4]

Following a washing step the cells are incubated for one hour with ascitic fluid of a FIPv infected laboratory cat. After the cells have been washed again they are incubated for an hour with Goat-α-Cat antiserum that has been labelled with FITC. Because FITC deteriorates when in contact with light, the cells are kept in the dark as much as possible from the moment the FITC is added. The cells are washed again before they can be examined using fluorescence microscopy.

immunoperoxidase staining (IPOX)

This technique can be used both on slides and with cell-culture plates, because the results can be viewed with a regular microscope. The cells are first incubated with goat serum to block all non-specific binding of antibody. Then the cells are incubated with the same antibody as in the IFT staining (ascitic fluid of a FIPv infected laboratory cat). After washing the cells, biotin-labelled Goat-α-Cat antiserum is added and left to incubate for half an hour. Then the biotin is labelled with streptavidin in another thirty minutes. Afterwards the peroxidase substrate is added and within 10 minutes colouring becomes visible.

growth curve of FIPv on Dendritic Cells

As Dendritic cells had never before been infected with FIPv in this laboratory it was important to measure and describe the kinetics of virus production. Therefore an infection experiment was devised where Dendritic Cells are plated into a 24 wells cluster at fifty thousand cells per well. Four wells are filled, of which three are infected with FIPv at a MOI of 50 $TCID_{50}$ per cell. FCWF cells are used as a control with similar cell concentration and MOI. After incubating for three hours, the cells are washed four times. A sample is taken directly after the cells are washed, and then every two hours for the next twelve hours. After that, a sample is taken every day for seven days. The samples are kept at –20°C until they are titrated in a $TCID_{50}$ assay.

induction of apoptosis in Thymocytes through FIPv-infected Dendritic Cells

The capacity of infected Dendritic Cells to induce apoptosis in Thymocytes should be measured in an infected co-culture. In order to distinguish Thymocytes from Dendritic Cells, the co-culture can be labelled for CD4 and CD8.

Apoptosis is visualised with the TUNEL method, and a positive control of apoptotic Thymocytes is provided.
Due to constraints in the available fluorescent labels, interference of signal might be a problem when using all three markers. Therefore it was decided to use only α-CD4 with the orange phycoerythrin (PE) label, and TUNEL with the green fluoro-isothiocyanate (FITC) label. This way we can only identify part of the Thymocyte population, but as we are interested in a phenomenon that is expected to affect CD4-expressing and CD8-expressing cells equally, this should not matter.

positive control: apoptosis caused by hydrogen peroxide

In order to have a positive control, a substance was needed that induces apoptosis. Hydrogen peroxide is known to cause apoptosis but no definite effective concentration was known for Thymocytes.[8] Therefore, an experiment was devised with different concentrations of H_2O_2. On a 96 wells plate Thymocytes are distributed at 50000 cells per well. They are incubated for 24 hours with 0, 0.5 or 1 mM H_2O_2. After incubation the cells are labelled with α-CD4 and TUNEL and analyzed with a FACS machine.

labelling for CD4 and CD8[4]

First, the cells are plated into a 96 wells plate at $1*10^7$ cells per well and subjected to one of the experiments described. After this treatment the cells are washed: the plate is centrifuged at 1200 RPM and the supernatant is removed. The cells are resuspended in FACS buffer (PBS0 with 1% BSA and 0,1% NaAz) and again centrifuged and relieved of their supernatant.
The solution of fluorescent labelled antibodies can now be added. Note that after addition of any fluorescent label the solution has to be kept in dark conditions as much as possible. It is possible to add both α-CD4 and α-CD8 at the same time. The plate is left to incubate for 30 minutes and then washed. If no other labelling is required the cells must be resuspended in FACS buffer and can be stored at 4°C until they are analysed.

detection of apoptosis: TUNEL

The acronym TUNEL stands for TdT-mediated X-dUTP nick end labelling. TdT stands for terminal deoxynucleotidyl transferase. X-dUTP is a labelled nucleotide. The label can be for instance biotin or fluorescein. The enzyme TdT tacks a nucleotide onto every 3' end of a DNA strand. By supplying labelled nucleotides the number of ends can be made visible. In an apoptotic cell the DNA strands are fragmented, creating many more ends. Apoptotic cells will 'light up' among the surrounding viable cells. [3, 13, 20]
If any other labelling is to be done, it has to be carried out before fixation, as fixation denatures proteins after which they may no longer be recognized by immunoglobulins. In the present experiment the cells were labelled with CD4 as described above.
The cells are now ready for fixation. Per well 100 µl of 4% Paraformaldehyde is added. This is incubated for 60 minutes, after which the plate is washed. The next step is to permeabilise the cell membranes by incubating for 2 minutes with a solution of 0.1% Triton-X100 and 0,1% citrate in MilliQ, followed by another washing step.
The control well is now incubated for 10 minutes with DNase I and then washed.
The TdT enzyme is mixed with the nucleotide solution at a ratio of 1:9. The mix is added to all wells except the negative control, which receives nucleotide solution without the enzyme. The plate is incubated for 60 minutes at 37°C. After having been washed twice the cells are resuspended in FACS buffer and kept at 4°C and in the dark until they are analysed with a FACS machine.

Infected co-culture of Dendritic Cells and Thymocytes

The ratio of Thymocytes to Dendritic Cells in co-culture is ten to one. Five thousand Dendritic Cells for fifty thousand Thymocytes per well of a 96 well plate are used. The medium is supplemented with IL-2 for the benefit of the Thymocytes. The number of wells used in a single experiment is largely dictated by the amount of Dendritic Cells that are harvested. One series of wells is incubated for three hours with 50 $TCID_{50}$ per cell of FIPv while the other is left uninfected. After having been washed four times, the cells are given fresh medium and left to incubate for 48 hours.
The cells are labelled with α-feline CD4 as described above.

RESULTS

Dendritic Cells

In working with Dendritic Cells the researcher must be prepared to have only small amounts of cells available. During freezing, the vials were filled with circa $1*10^7$ cells. Usually, two vials are thawed at the same time to insure that an adequate amount of DCs can be produced.

conventional culturing

In this laboratory, the typical yield of DCs per vial of bone-marrow used is about $2*10^5$ cells. In the thawing process, the cells are fragile and will be damaged by any rough handling. In later cultures, the yield approached typical numbers.(fig.1)

Selection for CD14 using MACS

The bone-marrow purified for cells expressing CD14 yielded cells similar in phenotype to the cells harvested from conventional culturing. The amounts harvested were however a factor ten lower than in regular culturing. (fig.1)

production of virus-stock

The virus stock produced reached a $TCID_{50}$ of $1.78*10^7$. This is much lower than the stock that is most used in this lab, of which a $TCID_{50}$ has been measured of $4*10^7$. In the experiments described, either the newly produced stock was used or the regular lab-stock.

determining an appropriate MOI

The infection experiments of DCs yielded very few infected cells. Due to the variable number of cells present on the slides it was difficult to quantify how many cells were infected. Of the FCWF, about 5000 cells were prepared per slide, but only about 10-20 positive cells could be counted per slide. It was difficult to see if there were any negative cells present. As FIPv generally approaches 100% infection of FCWF cells after 10 hours of incubation, this leads to the suspicion that many cells were lost in the staining process.
In the DCs, there is a difference between the MOIs used: MOIs 0 – 10 yielded no positive cells, MOIs 15-50 yielded 1-2, and MOI 100 yielded 1-4 positive cells.

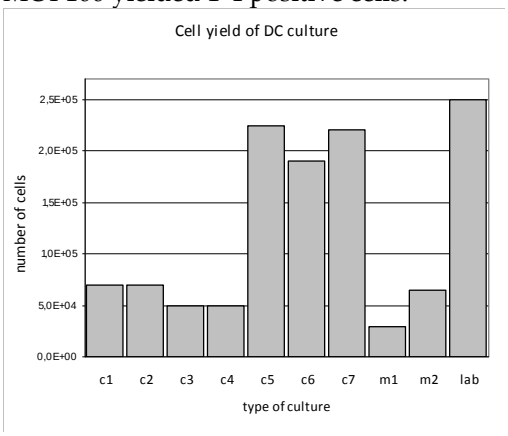

Figure 1. Cell yield of DC culture. c#: conventional culture, m#: MACS culture, lab: typical result

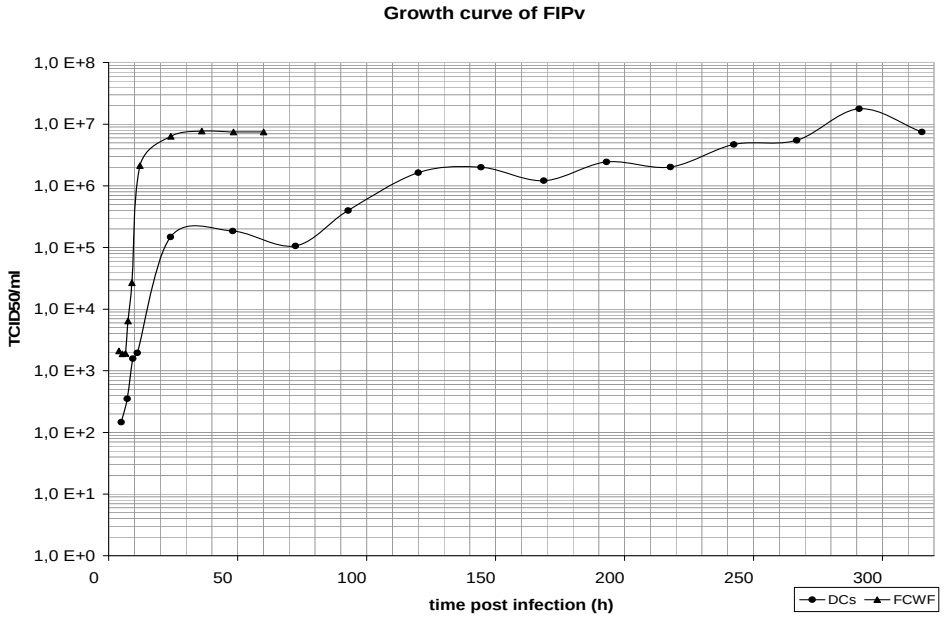

Figure 2. Growth curve of FIPv on FCWF cells and DCs

Calculating percentages from these numbers is not helpful, and in the end a MOI of 50 $TCID_{50}$ per cell was chosen for use in the experiments that followed.

growth curve of FIPv on Dendritic Cells

When infected with a MOI of 50 $TCID_{50}$ per cell, the DCs started to produce virus and reached a plateau after approximately 130 hours. This plateau reached titres of up to $1.8*10^7$ $TCID_{50}$ per ml. In comparison, FCWF cells reached a plateau in 24 hours and a titre of $7.7*10^6$ $TCID_{50}$ per ml. (fig.2) A mathematical correction was performed on the data to compensate for changes in volume caused by sampling. The fact that the chart does not show a smooth line may be due to greater differences in volume than was compensated for, and to normal inaccuracies in sampling and $TCID_{50}$ testing.

induction of apoptosis in Thymocytes

apoptosis caused by hydrogen peroxide

When assessing FACS results, it is customary to use a *gate*. This is a tool to select a subgroup of events from the total measured. Usually, the gate is chosen based on characteristics of size and complexity of the cells. The use of a gate results in cleaner data. However, in this particular experiment, such a gate will interfere with the results. As the object is to measure apoptosis, we need to see both apoptotic and viable cells in the results. These two groups of cells differ greatly in both size and complexity.
As shown in table 2, the positive control shows adequate staining for CD4 (25%) and TUNEL (46%), with a large number of cells stained for both (19%). The undisturbed population of Thymocytes (0 mM H_2O_2) has a CD4 positive component of 30%, while the samples with 0.5 and 1 mM H_2O_2 have a decidedly lower amount (23% and 13%, respectively). In these three samples, the fraction of TUNEL positive cells rises from 13% through 18% to 29%. In all but the positive control, the number of double positive cells is less than 1%.
In the three samples without induced apoptosis, the fraction of CD4 positive cells differs greatly: positive control 25%, negative control 36%, and 0 mM H_2O_2 30%.
Figure 3 and 4 illustrate these numbers for the 0 mM H_2O_2 sample and the 1 mM H_2O_2 sample.

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The results of this experiment are summarised in table 3. Figures 5 – 8 give a visual representation of some of the samples. Forward Scatter (FSC), which roughly corresponds to cell size, is displayed on the X-axis, and expression of CD4 on the Y-axis. For legibility, only half of the events are shown in the figures.
In figure 5, the results of the Thymocytes alone are plotted. In this figure, three distinct groups of events can be found: one group of relatively large sized cells that express

Sample	number of events	% CD4 positive	% TUNEL positive	% double positive
+ control	74535	25.26	45.85	19.31
- control	100000	36.29	0.18	0.10
0 mM H_2O_2	100000	30.08	13.30	0.69
0.5 mM H_2O_2	100000	23.12	18.44	0.42
1 mM H_2O_2	100000	13.04	28.76	0.75

Table 2. Apoptosis caused by hydrogen peroxide (FACS data)

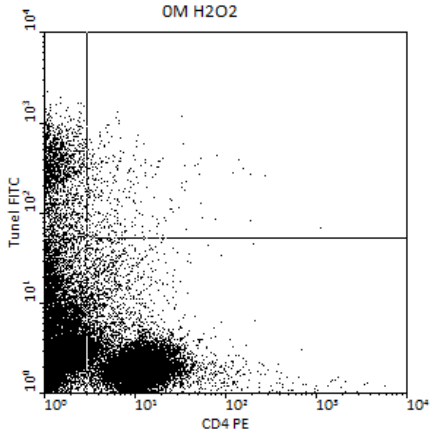

Figure 3. Dot plot of 50000 events of the apoptosis experiment with H_2O_2; 0 mM sample

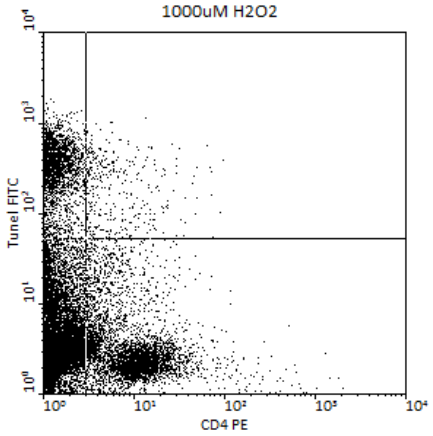

Figure 4. Dot plot of 50000 events of the apoptosis experiment with H_2O_2; 1 mM sample

CD4 (the upper right quadrant, 16%), another group of cells with roughly the same size that do not express CD4 (lower right quadrant, 38%), and lastly a group of cells and cell debris that have a smaller size and do not express CD4 (lower left quadrant, 46%).
In figures 7 and 8, which show DCs with Thymocytes, and FIPv- infected DCs with Thymocytes, respectively, we see the same groups again, with similar percentages. The group in the lower right quadrant is somewhat larger in comparison (49% and 51%), while the lower left group is smaller (30% and 28%). In the upper right quadrant – the CD4 positive group – the differences are smaller; the percentage has changed from 16% to 20% and 21%.
The most marked differences can be observed in the Thymocytes with H_2O_2 (figure 6). We can now see five groups of cells: two new groups seem to have originated from the groups in the upper and lower right quadrants and 'slid' into the lower left quadrant. This is supported by the fact that the upper right quadrant now contains only 3% of events, and the lower right quadrant has 17%.

The Thymocytes with FIPv have more cells in both upper right (19%) and lower right (45%), and a little less in lower left (36%).
One sample contained only DCs, to see how much the presence of DCs might interfere with measuring features of the Thymocytes. The DCs show 7% in upper right, 21% in lower right and 71% in the lower left quadrant. It seems that in this setup some Dendritic Cells fall into the categories that were described for Thymocytes. This means that in the co-cultures, some DCs interfere with the measurement of the Thymocytes. If we assume that DCs are not affected by being in co-culture, we can calculate the (assumed) true percentages of the Thymocytes.
In co-culture, there is one DC for every 10 Thymocytes. It follows that 1/11 of events must be generated by DC. We know that 7% of DCs fall into the upper right quadrant, and 21% in the lower right. Thus, in co-culture measurements, 0.64% of events in the upper right quadrant are caused by DCs, and 1,91% in the lower right. This seems negligible, but to be precise a new table can be created (table 4).

Sample	number of events	% in UR	% in LR	% in LL
Ts	100000	15.95	38.01	45.63
Ts H2O2	100000	3.28	16.53	79.04
Ts FIP	100000	18.65	44.90	36.12
Ts DCs	100000	20.46	49.01	30.12
Ts DCs FIP	100000	20.58	51.14	27.84
DCs FIP	18150	6.80	20.80	71.32

Table 3. Expression of CD4 in infected co-culture experiment; Ts = Thymocytes, UR = Upper Right Quadrant, LR = Lower Right Quadrant, LL = Lower Left Quadrant

Sample	%in upper right	% in lower right
Ts DCs	19.82	47.10
Ts DCs FIP	19.94	49.23

Table 4. . Expression of CD4 in infected co-culture experiment, corrected for presence of DCs; Ts = Thymocytes

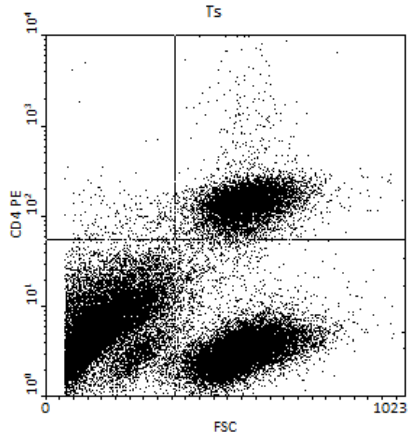

Figure 5. Dot plot of 50000 events of the infected co-culture experiment; sample of only Thymocytes

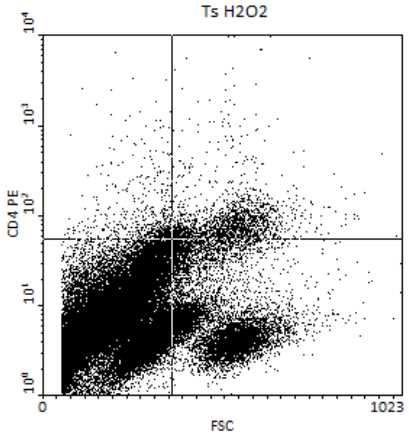

Figure 6. Dot plot of 50000 events of the infected co-culture experiment; sample of Thymocytes with 1 mM H_2O_2

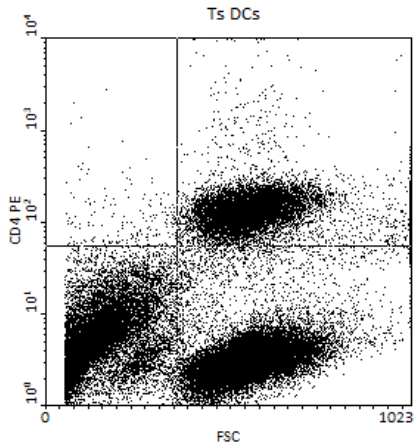

Figure 7. Dot plot of 50000 events of the infected co-culture experiment; sample of Thymocytes with Dendritic Cells

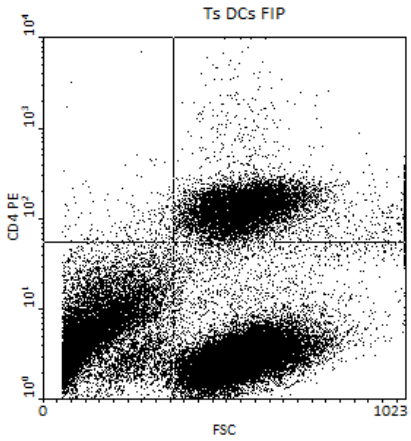

Figure 8. Dot plot of 50000 events of the infected co-culture experiment; sample of Thymocytes with Dendritic Cells and infected with FIPv

DISCUSSION

differences in cell yield between conventional DC culture and MACS

The difference in cell yield between conventional culture and MACS is remarkable. One might be tempted to conclude that not all DCs harvested from conventional culture arose from $CD14^+$ progenitors. However, we must not forget the fragility of newly thawed cells that has been mentioned above. It may be that the process of MACS itself leads to cell damage and thus less viable cells.

low number of infected DCs in spite of high MOI

In her report, Mak describes infection percentages of 100% for FCWF after 8 hours incubation. Bone-marrow derived DCs and Macrophages are 1% infected after 6 hours, and 40% after 16 hours.[19] In this experiment, the FCWF yielded only 10-20 positive cells of an expected 5000. This means that 99.6% of the expected positive cells were either not labelled correctly or lost. This illustrates the difficulties these results present for interpretation. If we assume that the technical problems for DCs were the same as for FCWF, the numbers of positive cells found correspond to 3-6 % of the population. This would fit with the results of Mak, however, the low numbers make this calculation highly unreliable.

apoptosis caused by hydrogen peroxide

When we compare the three untreated samples (positive control, negative control, and 0 mM H_2O_2), some marked differences in CD4 labelling are noted. One explanation for this phenomenon is that the DNase I treatment in the positive control somehow interferes with the labelling of CD4. However, this does not explain the difference between the negative control and the 0 mM H_2O_2 sample. It is therefore more likely that the difference is caused by signal interference in the detection of the different fluorescent labels: the labels used have some overlap in their spectrum.
It appears that for every % of TUNEL label measured, 0.25% to 0.5% of CD4 label disappears. If we now calculate the expected amount of CD4 (using the 0.5% disappearance rate) for 0.5 mM H_2O_2 and 1 mM H_2O_2, we must expect 27% and 22%, respectively. As this is still higher than the actual amount of CD4 found, there is another process that results in loss of CD4 expression. In the process of apoptosis cells change their appearance, and it is probable that this process also results in the loss of CD4 expression. Further support for this theory can be found in the positive control where the DNA is fragmented artificially. Many of the positive control cells are labelled both with TUNEL and for CD4, while in the other samples almost none are. As it seemed that loss of CD4 marked apoptosis just as well as fragmentation of DNA did, it was decided to forego the expensive and laborious TUNEL labelling procedure in the next test and label solely with α-CD4.

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The differences between normal Thymocytes and those treated with H_2O_2 seem to suggest that there is true apoptosis here: the cells diminish in size and lose their surface CD4.

There is a difference in CD4 expression between Thymocytes with and without FIPv of about 3%. This may be due to normal variation between Thymocyte populations. Table 4 shows the features of Thymocytes in co-culture corrected for the presence of DCs. The percentages are similar to those of Thymocytes in monoculture. The differences between the co-cultures with and without FIPv are smaller than those between the two monocultures of Thymocytes. This must lead to the conclusion that there is no apoptosis caused by the presence of FIPv in this setup.

possible causes of the absence of apoptosis in this experiment

While it may be tempting to conclude from this data that FIPv-infected Dendritic Cells are not the cause of T-cell depletion in cats suffering from Feline Infectious Peritonitis, there are too many variables still unexplored to make such a statement possible.
The most obvious impediment to such a statement is that Thymocytes are not T-cells, and a petri dish is not a cat. Besides that, at the time of these experiments, the 'DC' produced through MACS had not been sufficiently characterised. The growth curve had been performed on conventional DCs, and MACS DCs were used in the co-culture experiments. It is therefore not known if MACS DCs are infected with FIPv to the same degree as are conventional DCs, and this may influence co-culture results. Further research is needed to characterise cultured feline DCs obtained using the MACS technique.
The low rate of FIPv infection of DCs that could be achieved in these experiments suggests that FIPv infected DCs could not possibly have an effect on the entire T-cell population. However, in Measles virus infection, it has been shown that a low number of infected DCs can block the allostimulatory properties of the entire group. When added to co-culture with 10^4 uninfected DCs, as few as 10 infected DCs could completely block allostimulatory activity. If the infected DCs were treated to prevent release of infectious virus, 10^3 DCs were needed to achieve the same result.[12] While this means that the low infection rate does not have to be the reason for the absence of apoptosis in this experiment, it remains that these results are not what we had hoped to find. For reasons mentioned above, these results should not be taken as hard fact. They do, however, make the theory of FIPv infected DCs as the cause of T-cell depletion less likely. Other causes of T-cell apoptosis should be investigated.

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