The Effect of Influenza A Viral Infection on Dendritic Cells' Antigen Presentation

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Abstract

Influenza A virus (IAV) is one of the most contagious viruses to humans. Every year the seasonal influenza strikes worldwide, 500 000 deaths are estimated annually. A viral infection triggers the immune system to respond as a defence mechanism. The immune system is a complex system of cell types, all working to eliminate pathogens. The dendritic cells (DCs) are communicative cells that activate the most efficient immune cells, the T-cells. The DCs are the most crucial link to induce a full immune response; therefore it is of importance that the DCs antigen presentation is well functioning. It has been shown that IAV infection affects the antigen-presenting proficiency, especially on MHC-class I. The aim of this study was to get an indication of how proficient antigen presentation by IAV infected DCs is depending on time elapsed since the infection begun. Also to locate the virus particles in DCs infected for 4 hours. Expansion of flu-specific memory T-cells upon co-culture with IAV infected DCs was measured by a flow cytometer. It resulted in the conclusion that the monocyte-derived dendritic cells (MDDCs) proficiency in antigen presentation were affected by the infection. The induced proliferation of T-cells was low when MDDCs infected between 8 and 24 hours presented antigens. This is an indication of impairments in the antigen presentation pathways. The location of the virus after 4 hours of infection of MDDCs were in the membrane or the golgi apparatus and ER. In future studies the MDDCs could be infected up to 48 hours before fixation, to study if the antigen presentation recovers. Also looking further into the pathways of antigen presentation in DCs during an IAV infection to determine more precise where the impairment occurs could be of interest.

Contents

List of Abbreviations

IVA	Influenza A Virus
HA	Hemagglutinin
NA	Neuraminidase
TLRs	Toll-like receptors
PAMPs	Pathogen-associated molecular patterns
DCs	Dendritic Cells
MDDCs	Monocyte-derived dendritic cells
IL-4	Interleukin 4
GM-CSF	Granulocyte-macrophage colony-stimulating factor
MHC-class I	Major histocompability complex class I
MHC-class II	Major histocompability complex class II
PBMCs	Periphal blood mononuclear cells
SEB	Staphylococcal enterotoxin B
HI-IAV	Heat inactivated influenza A virus
PBS	Phosphate-buffered saline
p.s	Permeabilisation solution
b.s	Blocking solution
NMS	Normal mouse serum

1 Introduction

Influenza A virus (IAV) is highly contagious and causes, worldwide, approximately 500.000 deaths annually. Most humans have had "the flu" at some point, but the viral infection is most severe in those with a compromised or undeveloped immune system. Other than the seasonal influenza, large outbreaks and several pandemics have historically emerged whenever a new type of influenza virus has started circulating among humans. The most fatal influenza pandemic so far was the "Spanish Flu" in the early 1900s, consequently between 20 and 50 million deaths was estimated worldwide. [1]

Dendritic cells (DCs) are communicating cells in the immune system whose task is to innitiate a full immune response agaist pathogens[2]. This project is divided into two parts. The first part of the project addresses with the process of IAV infection of DCs and visualisation within DCs of the infecting virions. The second part of the project is about how dendritic cells' antigen-presenting abilities is affected by an IAV infection at different times.

1.1 Influenza A virus

There are three types of influenza viruses; A,B and C. There are various strains of IAV, but the basic structure is similar. The virion consists of viral-RNA surrounded by a lipid membrane. Hemagglutinin (HA) and neuraminidase (NA) are two major glycoproteins attached to the membrane. Sialic acid bound to lipids or glycoproteins on the host cell is the receptor for HA. HA is necessary for the entry of the virion into the host cell. NA is an enzyme which cleaves sialic acid allowing release of virions from the host cell. [3]

It is possible to heat-inactivate IAV to prevent replication in cells, incubating live virus for 30 min at 56 degrees celsius. Heat-inactivated IAV (HI-IAV) is commonly used when studying input virus in comparison to replicating virus, as HI-IAV retain functional viral

proteins but are no longer infectious due to damaged viral RNA.

1.1.1 Influenza A Virus Infection

Viral entry is induced by viral HA binding to sialic acid on the cell membrane. Once the viron has entered the cell it aims for the nucleus where a mass-replication process of the viral-RNA starts via viral-RNA-dependent-polymerase. An illustration of described steps is presented in Figure 1, steps 1-3b. [4]
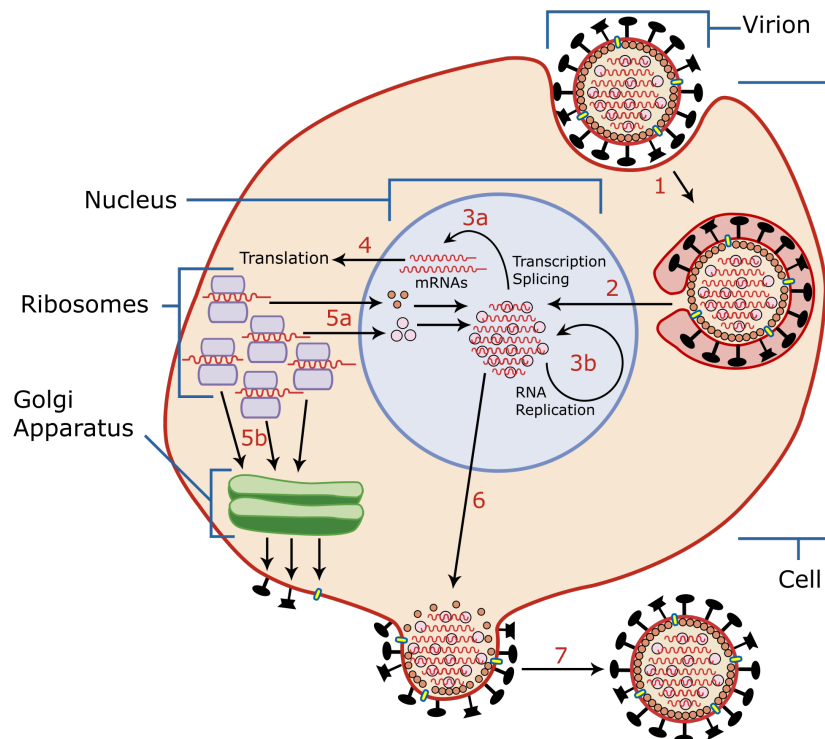
Figure 1: Viral infection of a cell step by step [1]

The replicated viral-RNA either assembles with viral proteins and exits the cell as new virions, thus spreading the infection to other cells (step 6, Figure 1), or transcribes splicing into mRNA (step 3a, Figure 1) and translates in the ribosomes (step 4, Figure 1) which results in defects in proteins and crucial processes in the cell (step 5a, Figure 1). The viral-proteins can also be sent to the cell surface via the golgi Apparatus (step 5b, Figure

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[1]Wikipedia Commons. *Virus Replication*. [published 05-03-07; cited 13-07-16] Avaliable from: `https://commons.wikimedia.org/wiki/File:Virus_Replication_large.svg`

3

1). Eventually the infection of the cell will lead to cell death as the virus takes over the host cell machinery. [4]

1.2   The Immune System

The immune system is the body's defense against pathogens. It is divided into two main parts; the innate and the adaptive immune system. The innate immune system is not as specific as the adaptive. For example, toll-like receptors (TLRs) on innate immune cells recognizes and binds to pathogen-associated molecular patterns (PAMPs) and starts a immune response based on a pattern, not a specific pathogen. The adaptive immune system is not as fast as the innate, but more sophisticated in its response. The innate consists of the granulocytes, dendritic cells and monocytes whileas the adaptive mainly consists of lymphocytes and NK-cells. An overview of the immune cells is presented in Figure 2. Antigens are carried by certain immune cells to the lymph nodes called antigen-presenting cells and introduced to the lymphocytes. Once activated, the lymphocytes target the pathogens specifically and thereby more efficiently than the innate immune cells. [2]

An indication of a working immune system is emitted cytokines. Cytokines are small proteins released by cells that affects interactions and communications between cells. Examples of cytokines are IFNg, IL-2 and TNF.[5]

The key to an effective elimination of pathogens is a working system of communicating cells, as mentioned above an activation of lymphocytes is required. The communication between the innate and the adaptive immune systems is mainly handled by monocytes, macrophages and dendritic cells. These cells are activated upon recognition of pathogens by TLRs on their cell surface. The lymphocytes consists of B-cells and T-cells. Activated B-cells can become memory-B-cells, that are long-lived and ready to start producing specific antibodies as soon as a pathogen reinfects. T-cells are mainly divided into cytotoxic-
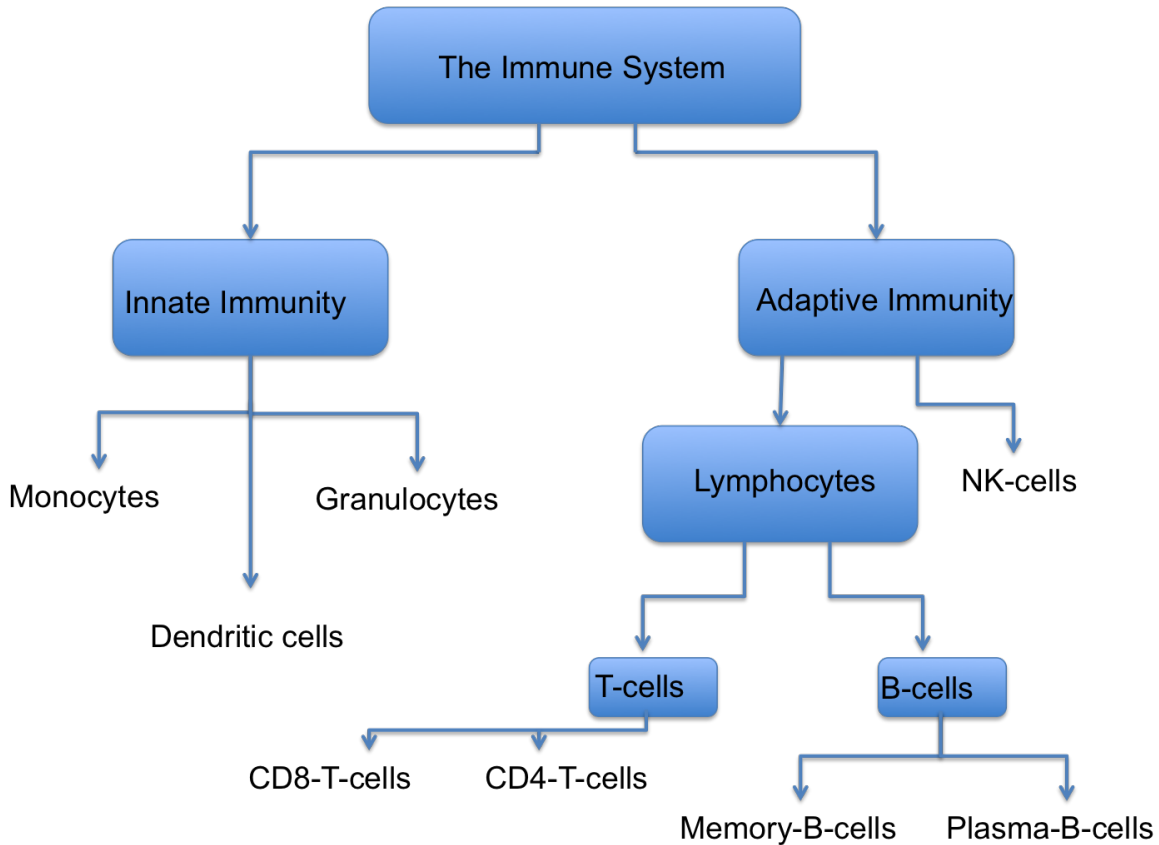
4

Figure 2: Overview of the innate and adaptive immune cells.

T-cells (killer-T-cells, CD8-T-cells) and helper-T-cells (CD4-T-cells). Cytotoxic-T-cells are the body's most efficient pathogen-destroying cells. Helper-T-cells activates other cytotoxic-T-cells but also B-cells. An activation of the lymphocytes induces the adaptive immune response so the T- and B-cells can fight the pathogens. T-cell proliferation is another consequence of the antigen presentation, the T-cells starts dividing to increase in number. [2]

1.2.1 Dendritic cells

DCs are communicative cells linking the innate and the adaptive immune systems. Their ability to regulate responses is unique due to the fact that they are able to regulate immunity in addition to inducing it. DCs are located wherever a pathogen has the possibility to enter the host, for example the skin, airways and lymphoid. DCs act as to sentinels, they scan everything passing by carefully to detect pathogens. Once a pathogen is de-

tected the DCs start moving through the lymphoid tissue towards regions with T-cells. The T-cells recognize the antigens and thereby gets activated. In addition, DCs activate the antibody production in B-cells that can neutralize the pathogens. DCs are a crucial part of the immune system since the links provided between antigens and lymphocytes are of importance. Without the link provided by DCs, the most efficient defence against pathogens remains unactivated. [6]

Differentation of monocytes can be steered into a type of DCs called monocyte-derived dendritic cells (MDDCs) by adding granulocyte-macrophage colony-stimulating factor (GM-CSF) combined with interleukin 4 (IL-4). GM-CSF induces the differentation and IL-4 triggers the specific differentation into DCs. In approximately five days the monocytes have completed the differentation into MDDCs. MDDCs are frecuently used in experiments, since the DCs in the blood are relatively few and harder to isolate.[7]

1.2.2   Presentation of Antigens by Dendritic Cells

DCs are professional antigen presenting cells, the uptake of antigens are enhanced by a variety in receptors and these antigens are presented on major histocompability complex (MHC) which are recognized by lymphocytes. There are two types of MHC, MHC-class I and MHC-class II. CD8-T-cells are activated by MHC-class I and CD4-T-cells by MHC-class II. [8]

Phagocytosis of pathogens and detection of pathogenic patterns by the TLRs on the DCs, induce the activation process of DCs as antigen-presenting cells. The maturation is approximated to take about 24 hours. Thereafter, processing of the content of the endocytic vesicles into MHC-peptide complexes that are sent to the cell surface. Peptide-MHC complexes are recognized by T-cells and the immune response is activated. [9]

For illustration of the pathways for antigen-presentation in DCs see Figure 3. There are

two direct presenting pathways, the MHC-class I pathway and the MHC-class II pathway. Classically, MHC-class II can present exogenous antigens, meaning CD4-T-cells are activated by DCs that have acquired antigens from other dead or dying cells, see Figure 3. The MHC-class I pathway presents endogenous antigens, meaning the DCs needs to be infected to be able to present. CD8-T-cells are activated by peptide-MHC-class I. All signaling pathways include the ER and golgi apparatus as a part of the antigen processing since that is were the viral-peptides binds to MHC. [10]

Another signaling pathway in DCs is called cross-presentation. The DCs have the ability to also present exogenous antigens by efficiently extracting extracellular peptides from dead and infected cells and presenting them on MHC-class I, see Figure 3. Cross-presentation is preferable in specific types of cells, for example DCs, since they tend not to be infected by viruses to the same extent as other celltypes. When studying IAV the highest extent of infection is in the epithelial cells. DCs have a unique ability to contain infecting virus, once a virion has entered the DC it is not able to leave. [2] The pathway of cross-presentation of antigens activates cytotoxic-T-cells since the peptides are presented as MHC-class I complexes. Thus, cross-presentation is, in comparison to other antigen-presentation of exogenous antigens and activation of CD8-T-cells (MHC-class II), more efficient[11].
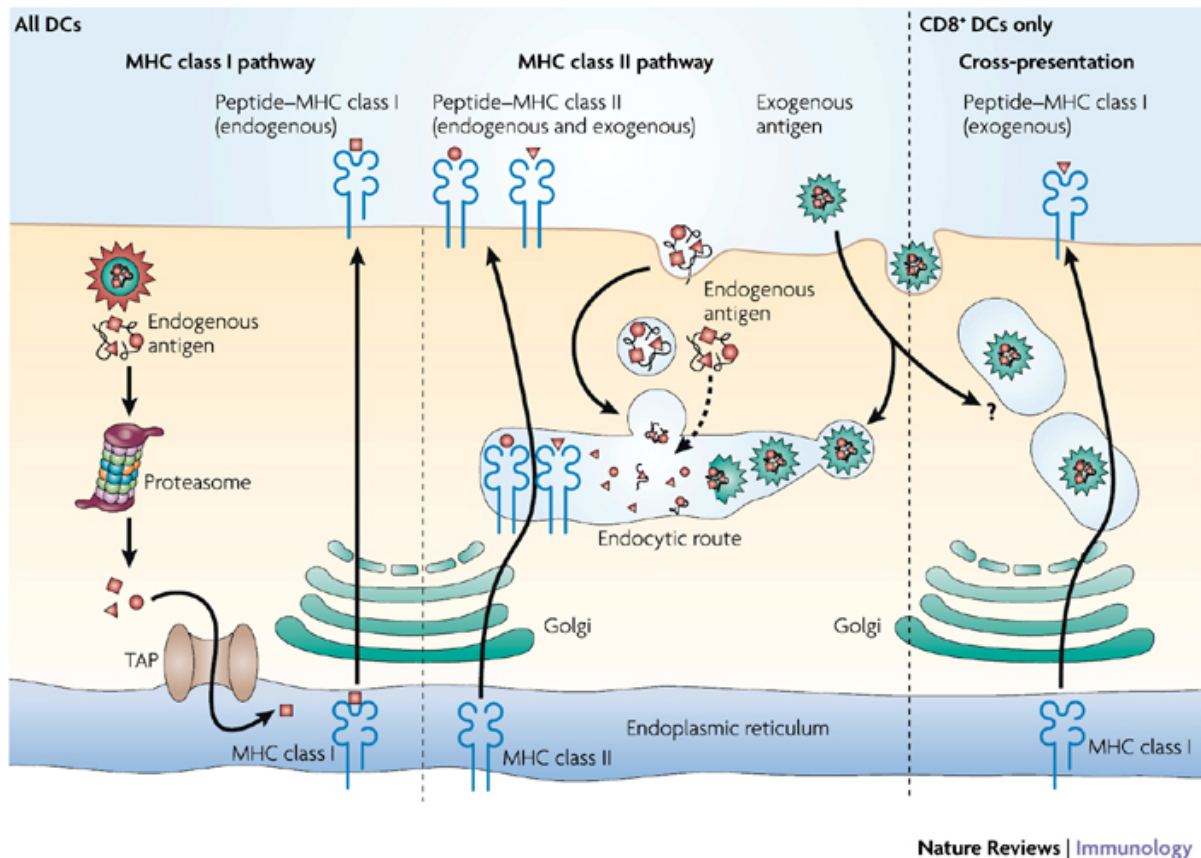
Figure 3: Overview of antigen-presenting pathways; MHC-class I, MHC-class II and Cross-presentation in DCs. [2]

1.2.3 Presentation of Antigens by IAV Infected Dendritic Cells

Research regarding the functional relationship between antigen-presentation in human DCs and infection is limitied. It is known that IAV entering the DC triggers DC-maturation into antigen-presenting cells, as most pathogens do. DC's unique capacity to present antigens via MHC-complexes to activate CD4 and CD8-T-cells seems to be affected by a IAV infection. Uninfected DCs are estimated to be more than 300-fold more efficient at CD8 stimulation than infected DCs. As a consequence of IAV infection cross-presentation and direct presentation of antigens may be reduced noticeably. The virus selectively impairs the DCs ability to present antigens on MHC-class I to CD8-T-cells. In theory this conse-

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[2]Villadangos A. J, Schnorrer P. *Intrinsic and cooperative antigen-presenting functions of dendritic-cell subsets in vivo.*[Online] Nature Reviews Immunology. [published -07; cited 11-07-16]. Avaliable from: `http://www.nature.com/nri/journal/v7/n7/fig_tab/nri2103_F2.html`.

quently leads to a weaker immune response from the CD8-T-cells. [11]

Studies of bigger viruses than IAV indicated that certain viral proteins specialized in impairing the MHC-class I pathway by mislocalize or cause degradation of MHC-class I molecules. Different viruses seem to find ways to disturb the antigen presentation pathways by using the cells own structures, such as antigen processing trafficking pathways and protein degradation. [12]

1.3   Aim of the Study

The aim of the study is to measure the expansion of flu-specific memory T-cells upon co-culture with IAV-infected DCs as an indication of proficiency in antigen presentation.

The secondary aim of the study is locating the virus particles infecting dendritic cells at 4 hours of infection.

1.4   Background to Methods

1.4.1   Immunofluorescent Staining

Both methods used in this study are depending on the principles of immunofluorescence. Fluorescent molecules, fluorochromes, excites and starts emitting light when light of the right wavelength is absorbed. These are used as dye to stain certain molecular compounds as a marker for such as antibodies and proteins. [13]

When antibodies are tagged with flourescent dyes and bind to antigens, the antibody-antigen-complexes can be detected by exciting the dye. Indirect immunoflourescence is based on fluorescenceconjugated secondary antibodies that bind to primary antibodies. The primary antibody is chosen to target specific antigens, there are two kinds - monoclonal and polyclonal - which binds to one or several epitopes. Epitopes are the part of

an antigen that is recognized by the antibodies. Polyclonal antibodies thereby is more likely to bind to non-target structures. The secondary antibodies bind specifically to the primary ones, see Figure 4. Multicolor immunoflourescence with secondary antibodies is possible if the antibodies derive from different species. For example when adding a murine primary antibody a anti-mouse secondary antibody can be used since it will bind to everything deriving from mouse. [13]
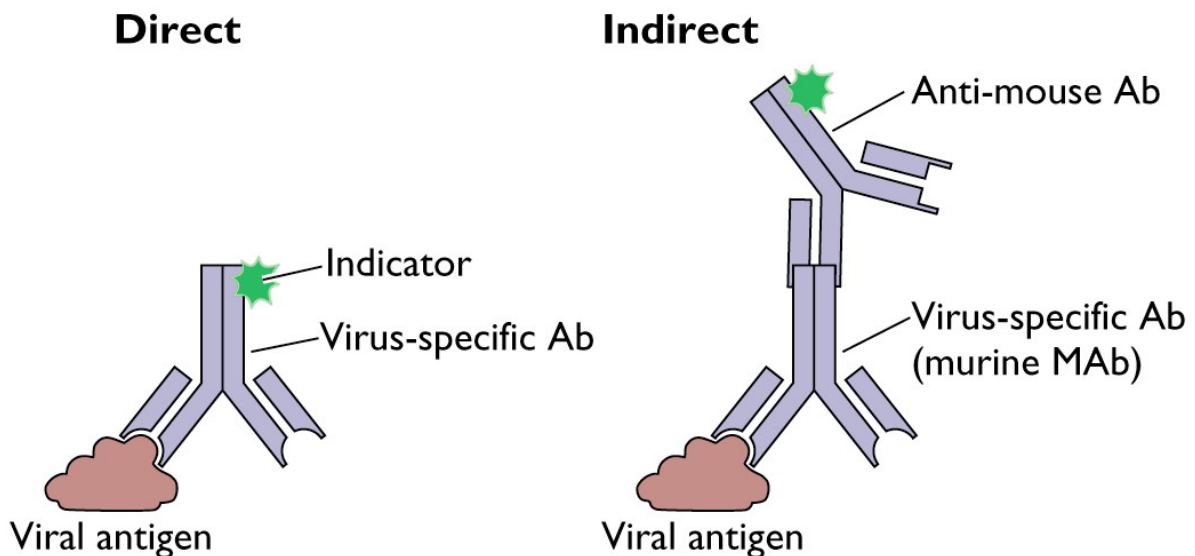

Figure 4: Direct immunoflourescence and indirect immunoflourescence staining principles. The indicator is the fluorescent part attached to the secondary antibody. Ab is an abbreviation of antibody. [3]

1.4.2 Confocal Microscopy

Confocal microscopes are based on the principles of fluorescence. A dyed specimen is placed in a laser beam which excites the flourescent dye. The flourescent light passes through an objective, a flourecence-barrier filter and a detector pinhole aperture before it is detected by a photomultiplier detector. The pinhole is a unique feature of confocal microscopes, it eliminates the out-of-focus light and flares from the specimen by the illumination through the pinhole. The photomultiplier and a special software on a connected

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[3]Racaniello V. *Detecting viral proteins in infected cells or tissues by immunostaining.*[Online] Virology blog. [published 20-09-2010; cited 13-07-16] Avaliable from: http://www.virology.ws/2010/09/30/detecting-viral-proteins-in-infected-cells-or-tissues-by-immunostaining/

computer creates images of the specimen, but it is also possible to watch the specimen using the confocal microscope ocularly. The specimen can be looked at in different planes aswell, meaning it is possible to see what is in the middle of the cell as well as on the top and bottom. Since the zoom function is very well-working it is not problematic to look at single cells in decent resolution. [14]

1.4.3   Flow Cytometry

Flow cytometry is a laser-based tool for analysis of different characteristics of cells. Flourescent intensity is measured to determine the expression of specific proteins both intracellular and on the cell surface. Different fluorescent molecules excites at different wavelengths, thereby a multi-parameter analysis of a single cell is possible. Cell poplulations can be separated and distinguished based on granularity and size as well. The flow cytometer has to be set up, before usage, with a set of controls, most importantly is to compesate spectral overlap of the different waveleghts. [15]

The cells pass individually through a laser beam, the light from the laser scatters and excites the labeled cell components. They become fluorescent and detectors will pick up a combinantion of fluorescent and scattered light, see left part of Figure 5. The data is analyzed by a computer and a special software connected to the flow cytometer. Fluorescence emissions express information about the cell component parameters while the scattered light informs about the size and granularity of the cell. [15]

Primary or secondary fluorochrome-labeled antibodies can be used for staining on the membrane. Intracellular staining and detection of secreted proteins are slightly more advanced processes that demand various permeabilization and fixation methods, see Appendix 2.
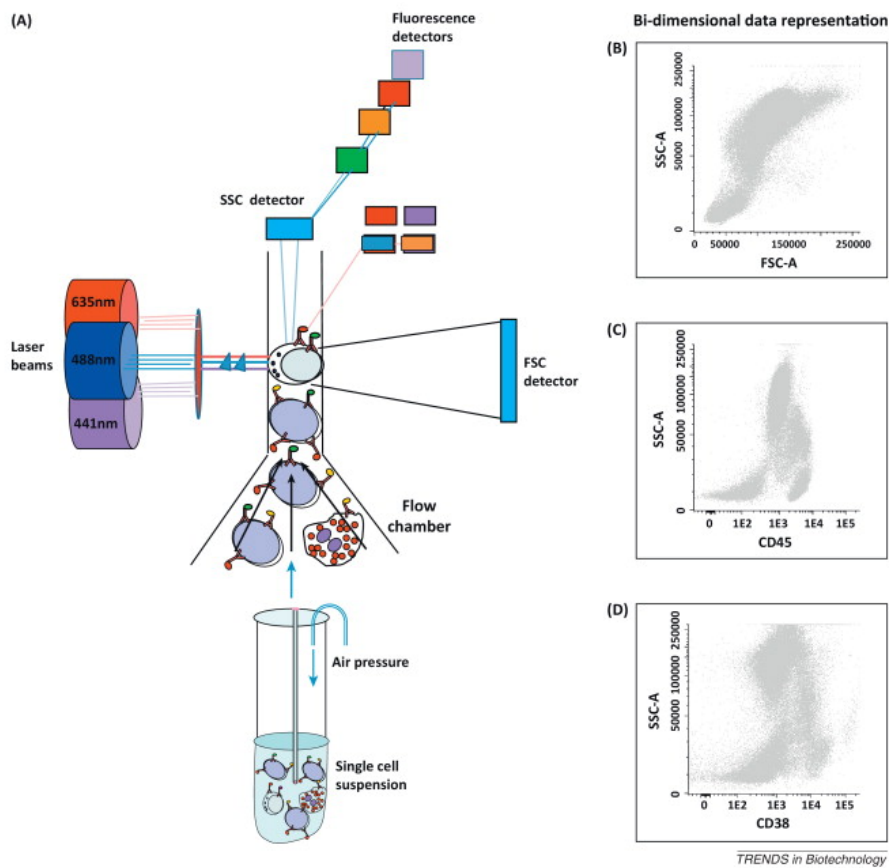
Figure 5: Overview of the principle of flow cytometry and examples of data analysis. The data representation charts is graded in different parameters. Different clusters of dots can be distinguished, each dot represents a single cell. [4]

The data analysis examples, in the right part of Figure 5, is graded to be interpreted as if half of the axes are negative and half positive. Thereby meaning that if a cell is located in the upper right corner it is positive for both parameters tested. The size and height parameters are expressed similarly in the data analysis tool, though they are not positive or negative since they are a product of measuring scattered light. [15]

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[4]Pedreira E. C, Lecrevisse Q, Van Dongen J.M. J, Orfao A. *Overview of clinical flow cytometry data analysis: recent advances and future challenges* Volume 31, Issue 7[published 07-13;cited 01-07-16] Avaliable from: `http://www.cell.com/trends/biotechnology/fulltext/S0167-7799(13)00094-2`

2 Method

2.1 Flow Cytometry

The different spectra of the lasers in the flow cytometer need to be regulated and the wavelengths separated before running any tests. Therefore calibration-tests were prepared and run to detect the different wavelengths of excitation of the different attached fluoresent molecules. For table consisting used dyes in compensation control see Appendix 3c.

2.1.1 Isolation of T-cells, Monocytes and Peripheral Blood Mononuclear Cells

Diluted blood from three different anonymous donors was recieved from Karolinska university hospital 28/6-16. The aim was to isolate peripheral blood mononuclear cells (PBMCs) in the buffy coat, more specifically the T-cells. By adding about 10 ml Ficoll and twice the amount diluted blood and centrifuge it for 25 minutes the different blood components were separated. Ficoll is a solution of polysaccharides used as a density gradient media to separate blood components, see Figure 6.
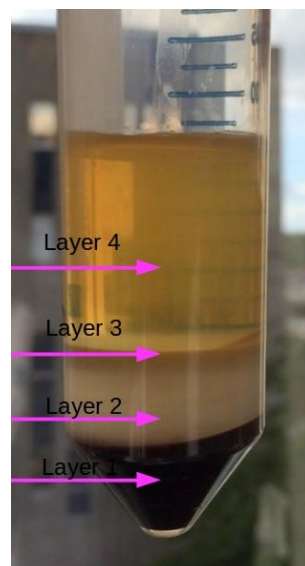

Figure 6: Example result of centrifugation of diluted blood and Ficoll. Layer 1 is red blood cells and granulocytes, layer 2 is ficoll, layer 3 is PBMCs and layer 4 is plasma.

Monocytes, PBMCs and T-cells were to be separately isolated from the PBMCs. T-cells were frozen for future use. Monocytes were incubated for six days in the presence of cytokines before they could be used in further experiments as MDDCs. PBMCs were incubated overnight. The following day, three test-tubes per donor of PBMCs were set up. One was left unstimulated, IAV was added into the second and SEB (Staphylococcal enterotoxin B) was added in the third tube (see protocol in Appendix 1). SEB was used as a positive control since it is categorized as a superantigen which causes a major immune response. The unstimulated test was used as a negative control as the PBMCs should not respond without a pathogen present. Intra- and extracelular staining of the PBMCs was added, (see protocol in Appendix 2).The purpose was to investigate whether the donor T-cells had an immune memory of the IAV from a previous infection or not. Percentage emitted cytokines in the T-cells was measured and used to estimate if the virus was recognized. The PBMCs were run through the flow cytometer and data collected.

A gating strategy is used as a tool in the analysis of the collected data from the flow cytometer. As seen in Figure 7 the gating strategy is applied step by step to exclude cells which are not of interest. There are excisting indications of the size of a lymphocyte (used in Figure 7, first chart) and in a single cell the height is in proportion to the width (Figure7, second chart). Also a staining determining if the cells are alive or dead is used in addition to distinguising T-cells (7, third chart), at last a factor separating CD8 and CD4 T-cells is studied (7, fourth chart). For full protocol see Protocol 2 in Appendix A. The final step to determine if the donated T-cells had a memory of the IAV strain was looking at the percentage of cytokines produced (IFNg, TNF and IL-2) in the IAV infected specimens.

2.1.2 Differentation into Monocyte-derived Dendritic Cells

IL-4 and GM-CSF were added to monocytes derived from the PBMCs from the donated diluted blood and the differentation process was thereby induced. The specimens were
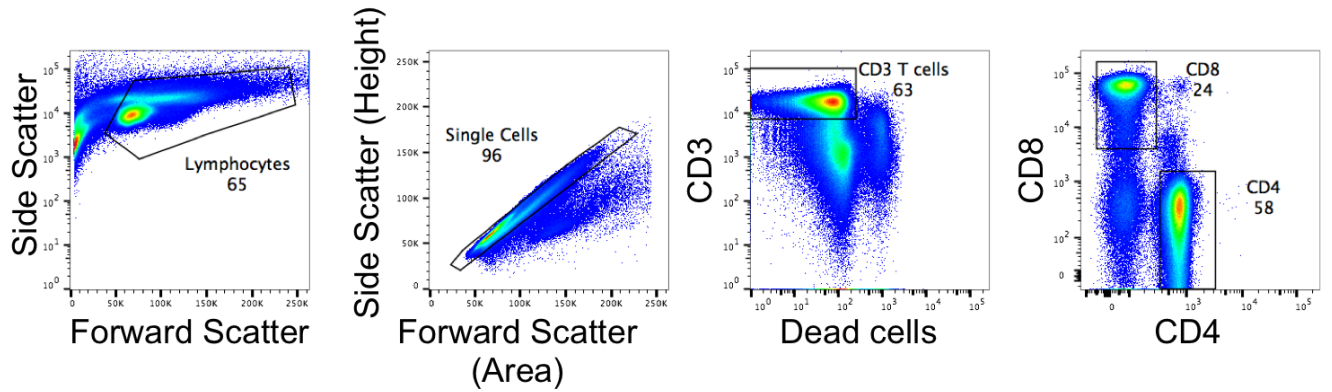
Figure 7: Gating Strategy to identify T-cells in PBMCs. In the first chart Lymphocytes are included, second single cells, third alive T-cells and in the fourth chart CD4- and CD8-T-cells are separated.

incubated for six days before the staining begun, see Protocol 1 in Appendix A.

2.1.3 CFSE Staining of T-cells and Co-culture Set-ups

CFSE staining of T-cells is used to measure T-cell proliferation when antigens are presented. T-cells were resuspended in PBS and thawt. CFSE dilution was added and the cells were incubated for 10 minutes. FCS and R10 was added, se Protocol 3a in Appendix A for detailed protocol.

MDDCs were added to the T-cells at a 1:30 ratio. SEB was added as a positive control. Tubes were stained for T-cell proliferation after 7 days of incubation and analysed by a flow cytometer. See details in Protocol 3a in Appendix A . The co-cultured tubes were run through a flow cytometer for analysis.

2.2 Confocal Microscopy

The stained MDDCs were studied in a confocal microscope. Three different coverslips were studied; 4h HI-IAV infected, 4h IAV infected and Unstimulated MDDCs. The cells were stained as described in Protocol 4 in Appendix A. Images were saved and ready for further analysis. Viral particles were expressed as green, cell membrane as blue and the

nucleus as grey.

2.2.1 Immunoflourescence Staining of Monocyte-derived Dendritic Cells

MDDCs were added, fixated, washed and permeabilized to five coverslips. The set-ups were: uninfected, 4 hour IAV infected, 4 hour HI-IAV infected. The permeablisation solution (p.s) consisted that of blocking solution (b.s) and Triton X-100. The b.s consisted PBS and goat serum that was later added directly to the coverslips to reduce non-specific binding, also trying to avoid imparing antibody-epitope bindings. Serums consists of different components binding to many different epitopes on the cell surface, but the primary antigens usually binds more specific which results in the primary antibodies breaking the serum-epitope bindnings. The serum-epitope bindings which were not broken by the primary antibodies thereby blocks secondary antibody-binding. For full protocol see Protocol 4 in Appendix A.

After incubation of the cells during 1 hour, primary antibodies (Rabbit PINDA; Mouse FK21) was added and the cells were incubated for 1 hour. B.s was used to wash the cells 4 times. Secondary antibodies were added (Anti-rabbit 488; Anti-mouse 555; See full protocol in Appendix A, protocol 4), thereafter the coverslips were incubated for 1 hour and washed 4 times with b.s., 100 µL 10 % normal mouse serum (NMS) in PBS was added to block for 15 minutes. 100 µL HLA-DR 647 diluted in p.s was added and the samples incubated for one hour. The coverslips were washed 4 times with b.s and twice with PBS. Washed meaning covered with the liquid and then removing the liquid. Drops of mounting solution was put on a glass slides before mounting the coverslips on the glass slides. The coverslips were dipped in water to remove salt and dried by touching filter paper. They were flipped and placed on the drop of mounting solution and at last put in the fridge for storage. For full protocol and set-ups of coverslips see Protocol 4 in Appendix A. Only the 4h and Uninfected coverslips were studied.

3 Results

3.1 Determining T-cell Memory of Influenza A Virus
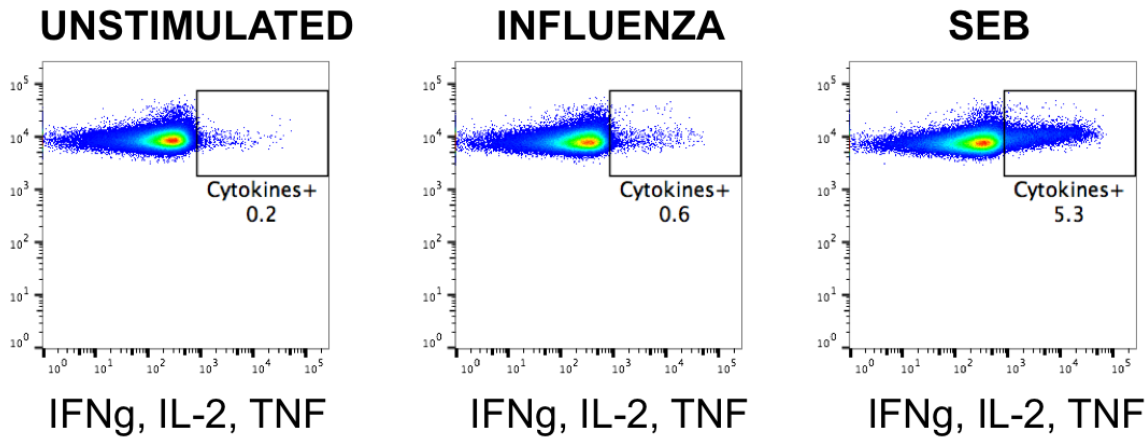
(a) Percent T-cells emitting cytokines (IFNg, IL-2, TNF) detected, results from one donor
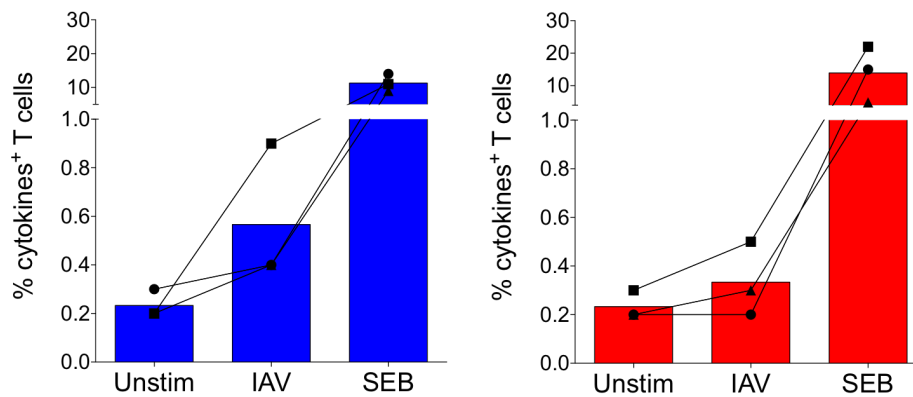
(b) Percent emitted cytokines detected from CD4- and CD8-T-cells

Figure 8: Results from PBMCs run in flow cytometer, detected cytokines in (a) is presented as the squares in (b).

Of the unstimulated PBMCs 0,2 % cytokines were emitted by the T-cells, see Figure 8a. 0,6 % cytokines were emitted by the T-cells in the influenza infected test and 5,3 % cytokines were emitted by T-cells stimulated with SEB, see Figure 8a

The donor represented by the square-symbol, see Figure 8b, has the biggest difference in percentage cytokines emitted when comparing IAV to Unstim PBMCs. The donors represented by triangles and circles in Figure 8b does not respond to the IAV stimulation.

3.2 Measuring CFSE Intensity to Estimate T-cell Proliferation and Antigen-presenting Function in Infected DCs

Figure 9: Measurement of percentage T-cells emissioning low levels of CFSE due to proliferation induced by antigen presentation by unfixed DCs (T-cells only included, SEB infected DCs, uninfected DCs, IAV- and HI-IAV infected DCs)

Figure 9 shows percentage T-cells proliferated until expressing low levels of CFSE staining as a reault of antigen presentation by unfixed DCs. One testube consisting of nothing but T-cells is included ("T-cells only"- staple in Figure 9). The presenting DCs represented by the different staples are SEB, IAV and HI-IAV infected, T-cells only and unifected DCs. CD8- and CD4-T-cells are separated in the results.

The staples of the CD4-T-cells expressing low levels of CFSE represent a percentage; SEB infected DCs 75%, IAV infected DCs 14% and HI-IAV infected 31%. The CD8-T-cell staples represent low emission of CFSE as following; SEB infected DCs 56%, IAV infected DCs 5,8%, HI-IAV infected DCs 19%. For additional images from the flow cy-

tometer of results and the raw data see Appendix B.
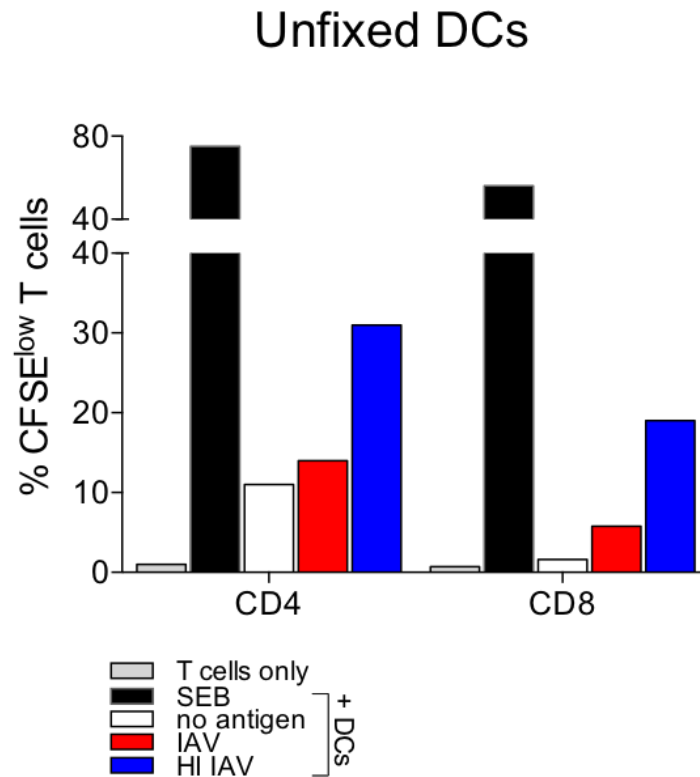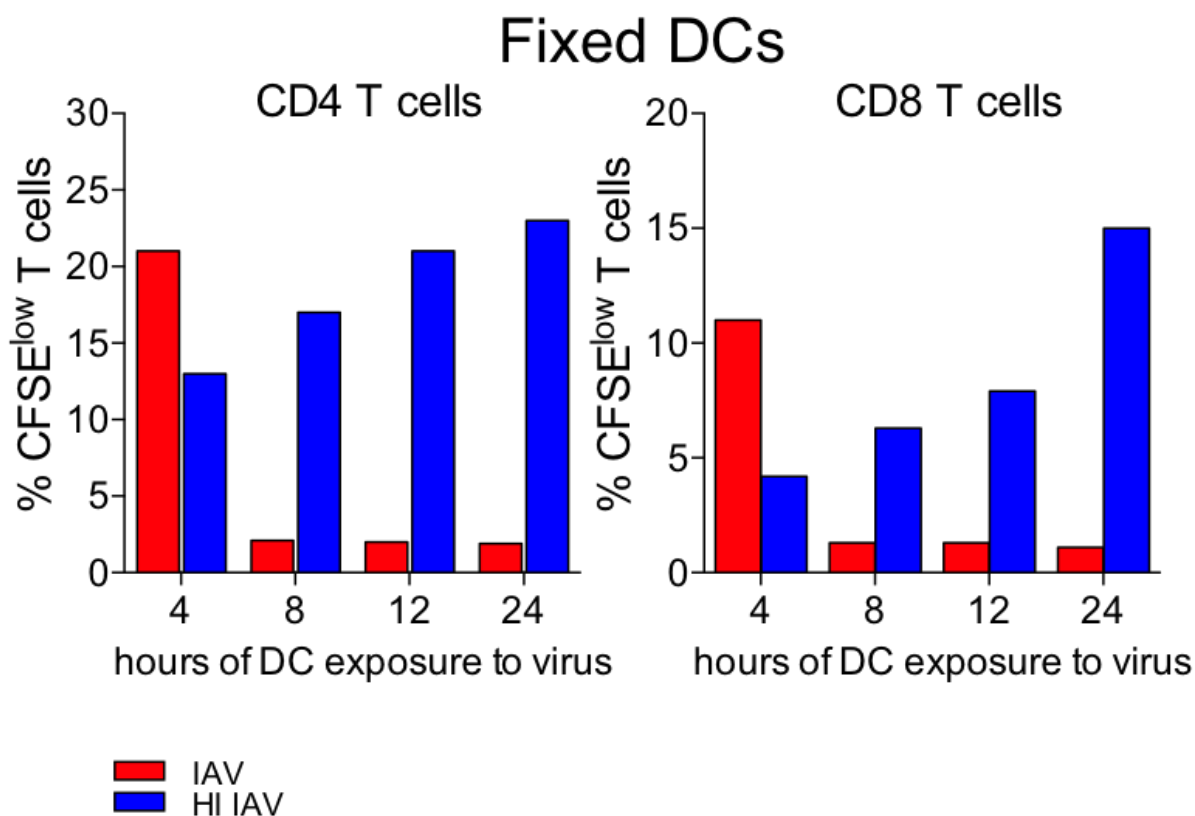

Figure 10: Measurement if percentage T-cells emitting low levels of CFSE due to proliferation induced by antigen presentation by DCs fixed at different times after viral infection (4, 8, 12, 24 hours) by IAV or HI-IAV.

In Figure 10 separate charts repressent CD4- and CD8-T-cells. The blue staples in both charts represent the co-cultures of T-cells and HI-IAV infected DCs and the red staples represent the co-cultures of T-cells and IAV infected DCs. The height of the staples represent the percentage of T-cells emitting lower levels of CFSE. For additional images from the flow cytometer of results and the raw data see Appendix B.

3.3 Confocal Microscopy

3.3.1 Overview of the Virus Location in Infected Dendritic Cells
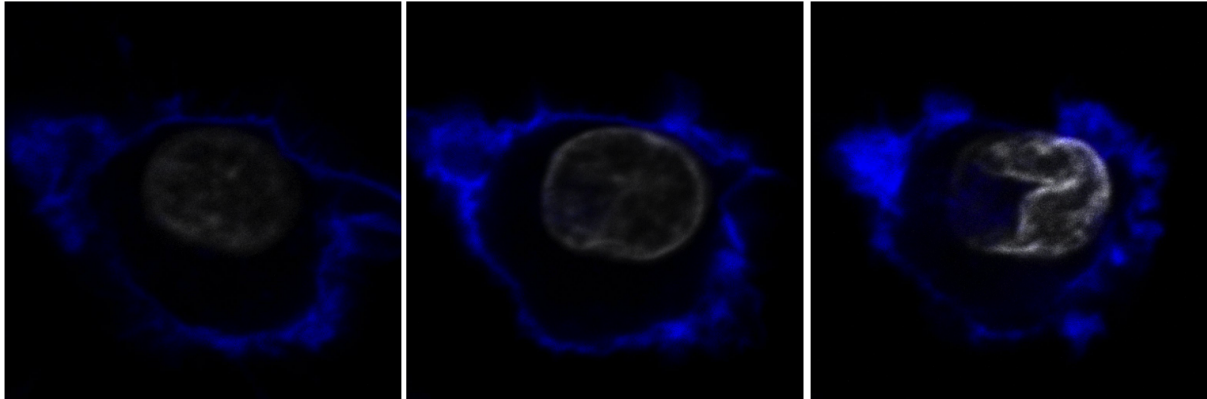
Figure 11: Uninfected MDDC included as a negative control

No virus detected in the uninfected MDDC, see Figure 11.
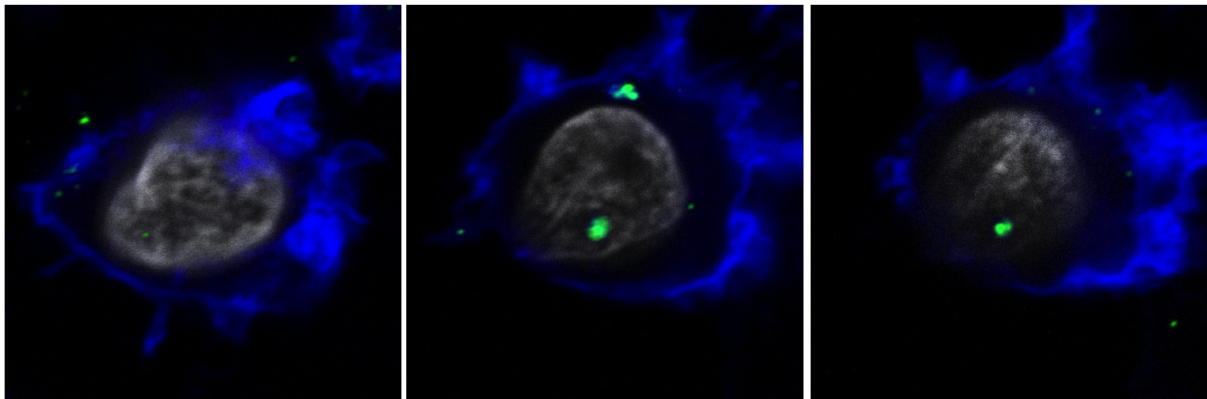
Figure 12: MDDC infected with HI-IAV for 4 hours

Some clusters of virus is detected in the MDDC, see Figure 12.
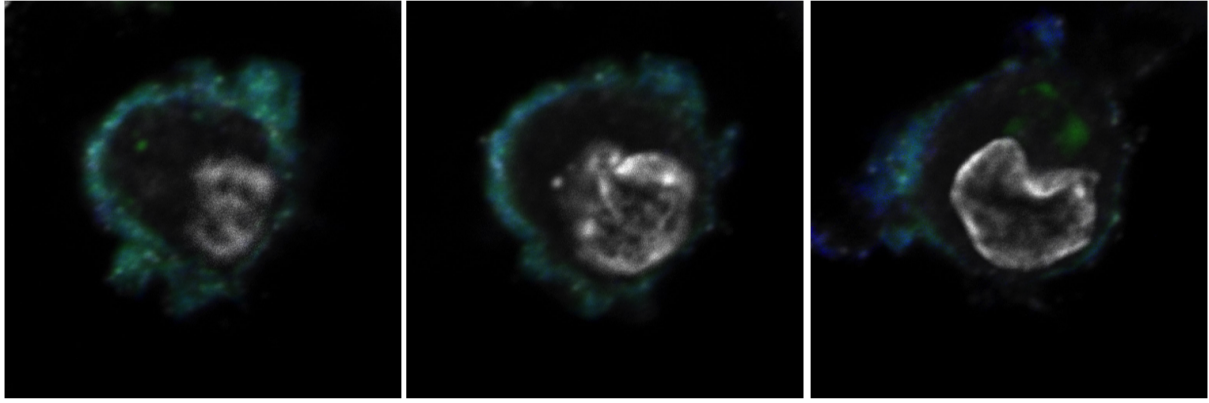
(a) MDDC expressing viral particles mostly on the cell membrane
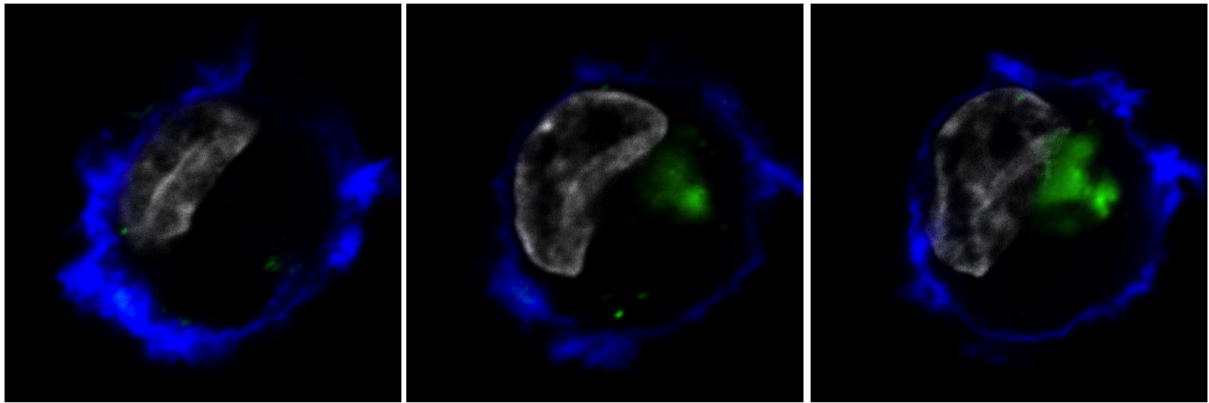
(b) Accumulation of virus in certain parts of the MDDC

Figure 13: MDDCs infected with IAV for 4 hours

Virus overlapping the membrane is detected in Figure 13a, the color of the membrane-virus overlaps are turquoise. In Figure 13b virus accumulate in a specific part of the MDDC.

4   Discussion

4.1   Influenza A Virus in Infected Dendritic Cells

The Mouse - anti-Mouse staining of ubiquitin, is chosen to be excluded in the results due to a small amount of collected data and poor relevance.

Since there are no detected virions in the uninfected dendritic cells, contamination is most likely not to have happened. In all Figures (11, 12, 13a, 13b) a cell is sorted in slides

in different planes; bottom, center and top of the cell.

In Figure 13a the color of the membrane is slightly different in comparision to in Figure 13b. The turquoise coloring in Figure 13a derives from virus located in the membrane, meaning the infection has advanced to the point when the newly synthezised virions are about to exit the host cell or being antigen-presented by MHC. Since the specimen is a dendritic cell there are mechanisms containing the virus within the cell, meaning it is stuck in the membrane.

The detected virus in Figure 13b has left the nucleus and presumably started clustering in the golgi apparatus and ER since it is a part of the antigen processing before the actual antigen presentation. The virus seems to have gone through the whole process of entering the nucleus, being replicated and leaving the nucleus at 4 hours of infecting the cell.

4.2 T-cell Response to Influenza A Virus

Two of the donors' T-cells did not respond to the added influenza virus (see Figure 8b, triangles and circles) which meant they were excluded from further experiments since the aim was to measure the expansion of flu-specific memory T-cells upon co-culture with IAV-infected dendritic cells as an indication of proficiency in antigen presentation.

Results shown in Figure 8a indicates an immune response on IAV since the percentage (0.6) is higher than the untimulated but lower than the SEB samples percentage of detected cytokines. The donor of interest is marked with squares, See Figure 8b, there is a distinguishable difference between the donor of interest's T-cell response to IAV in comparision to the others.

4.3 Dendritic Cell Induced T-cell Proliferation

The hypothesis was that the impairment of antigen presentation rather is a delay in antigen presentation. That was the reason why the cells were infected for 4 to 24 hours, to see if there is improvement in antigen presentation that could be measured by looking at T-cell proliferation. The generated data suggests that is not the case because although there is decent presentation at 4 hours, it crashes at 8 hours and does not really recover. It could be that it takes more than 24 hours for the recovery. Future studies could include MDDCs infected for more than 24 hours, but it could be problematic since the cells will begin dying from the virus infection.

The virus replicates using the host cell machinery which may result in the antigen-presenting functions being used for replication instead. No evidence of this theory has been established. Larger viruses has been shown to impair the antigen presentation pathways by viral proteins binding to MHC class I. IAV could interact similarly as other viruses. Regarding this, no generated data exists and it might be interesting to look further into how the antigen-presentation patways in detail is affected by IAV infection.

4.3.1 Unfixed Dendritic Cell Induced T-cell Proliferation

As seen in Figure 9, the 'T-cells only' test tube did not result in much proliferation of the T-cells. Probably since no antigens were presented that could induce T-cell activation. The result of the 'T-cells only tube' is used as a negative control which indicates that T-cell proliferation is not induced by other antigens than the ones presented by DCs. The positive SEB control resulted in frequent proliferation since the percentage of T-cells expressing low levels of CFSE is high, about 75% in CD4-T-cells and 56% in CD8-T-cells. The uninfected DCs ('no antigen') seem to induce some proliferation, especially in CD4-T-cells. There may be another exogenous antigen present in the test causing a response (presented via MHC-class II pathway). Though DCs can induce a T-cell response without specific antigen presentation.

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The antigen presentation by both IAV and HI-IAV infected DCs activates more CD4-T-cells than CD8-T-cells, see Figure 9. This further supports previous studies of IAV's effect on antigen presentation pathways, the antigen presentation by MHC-class I seems impaired. Furthermore, the MHC-class II patway seems to be impaired aswell. There is a difference in proliferation between T-cells activated by the HI-IAV infected DCs and the IAV infected DCs. The difference between IAV and HI-IAV in CD4-T-cell proliferation is 14 percentage and in CD8-T-cells 13,2 percentage. Both differentials are an indication of an impairment in antigen presentation probably caused by replicating virus.

4.3.2 Fixed Dendritic Cell Induced T-cell Proliferation

The results presented in Figure 10 indicates a difference in T-cell proliferation due to difference in antigen presentation by DCs depending on if they are infected by IAV or HI-IAV. The antigen presentation by HI-IAV infected DCs is not as instant as the presentation by IAV infected DCs. The 4 hour IAV infected DCs present antigens most efficiently to both CD4- and CD8-T-cells, both in comparison to DCs infected during longer time and in comparison to HI-IAV infected DCs (except the 24h fixation time). The activation of T-cells by IAV infected DCs is low except for the DCs fixated at 4 hours. This indicates a fault in the antigen presentation occuring between 4 and 8 hours after adding the virus.

4.4 Conclusions and Future Studies

The MDDCs proficiency in antigen presentation were affected by the IAV infection. At 8 hours of infection the ability to activate T-cells were low and it did not increase at the times included in the study. Fixation of cells after infection of IAV up to 48 hours could be studied in the future to see if the antigen presentation proficiency recovers. To understand why the antigen presentation proficiency of the DCs is affected, in future studies looking into the antigen presentation pathways in detail while infecting DCs with

IAV could be interesting.

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A Protocols

Following protocols were used in the method.

1. Isolating pan T cells using RosetteSep T cell enrichment kit

2. Testing IAC memory response in PBMCs

3. Presentation of IAV to memory CD4 and CD8 T cells by DCs

4. IF Staining of MDDCs infected with IAV/X31

1

Week 26

Isolating pan T cells using RosetteSep T cell enrichment kit

Donor 276, 277, 278

1. Add 500µl RosetteSep (T cell kit) to 10 ml of blood. Add 100µl RosetteSep (Monocyte enrichment kit) to 10 ml of blood. Incubate for 20 min at RT.
2. Dilute blood 1:1 with PBS to make up to 20 ml of blood.
3. Layer on top of Ficoll (10 ml ficoll + around 20 ml blood)
4. Spin for 25 min at 2200 rpm, no brake.
5. Generously remove the interface.
6. Wash cells in PBS, 1200 rpm, brake 7.
7. RBC lysis buffer 5 min. (if needed)
8. Wash cells in PBS, 1200 rpm, brake 7.
9. Count cells:

   PBMCs, Donor 276:   x 10^6 (from 5ml buffy coat)
   Monocytes, Donor 276 :   x 10^6 (from 25ml buffy coat)
   T cells, Donor 276:   x 10^6 (from 10ml buffy coat)

   PBMCs, Donor 277:   x 10^6 (from 5ml buffy coat)
   Monocytes, Donor 277:   x 10^6 (from 25ml buffy coat)
   T cells, Donor 277:   x 10^6 (from 10ml buffy coat)

   PBMCs, Donor 278:   x 10^6 (from 5ml buffy coat)
   Monocytes, Donor 278 :   x 10^6 (from 25ml buffy coat)
   T cells, Donor 278:   x 10^6 (from 10ml buffy coat)

10. For monocytes:
    - Resuspend in fresh R10 at a concentration of 0.5x10^6 cells/ml.
    - Add 25 ml of cells per flask with 40ng/ml GM-CSF & 40ng/ml IL-4.
    - Incubate for 6 days.
    - Change media and cytokines on day 3.

11. For PBMCs:
    - Incubate overnight unstimulated, 10 µg/ml SEB or 0.6 MOI IAV.
    - Add 10µg/ml BFA after one hour.
    - Stain for intracellular cytokines the next day.

12. For T cells:
    - Freeze the rest of the cells in freezing medium (90% FCS + 10% DMSO).

2

Week 26

Testing IAV memory response in PBMCs

- Wash in 3ml PBS 2% FCS 5min 1500rpm.
- Add 5 µl Live/Dead Blue (1:40 in PBS) for 5 min at 4°C.
- No wash. Add 5 µl FcR block (Miltenyi) to all tubes, 5 min at 4°C
- No wash. Add surface Abs for 15 min at 4°C.
- Wash in 3ml PBS (to remove FCS) 5min 1500rpm.
- Fix in 100µl Fix/Perm (eBioscience, dilute 1:3) at RT for 20min.
- Wash in 2ml Perm buffer (eBioscience, dilute 10x in H_2O) 5min 1500rpm.
- Add intracellular ab for 15 min at 4ºC.
- Wash in 2ml Perm buffer 5min 1500rpm.

Canto T cell cytokine panel:

Laser	Fluorochromes	Marker	Per Test (µl)	Total (n=9)
Blue 488	PerCP Cy5.5	CD8	2	
Blue 488	FITC	CD4	5	
Blue 488	PE Cy7	CD45 RA	1	
Blue 488	PE	CCR7	3	
Red 640	APC Cy7	CD3	0.25	
Red 640	APC	IFNg	1	
Red 640	APC	TNF	1	
Red 640	APC	IL-2	1	
Violet 405	AmCyan	Live/Dead Aqua	5 (1:20)	

* Intracellular, do not add to master mix!

3a

Week 27

Presentation of IAV to memory CD4 and CD8 T cells by DCs

160703
MDDC d277: 6×10^6 cells

Infection of DCs
7 pm (Sunday)
- Infect 0.6 MOI live or HI IAV/X31 to 500.000 MDDCs per tube, 24 h.
7 am (Monday)
- Infect 0.6 MOI live or HI IAV/X31 to 500.000 MDDCs per tube, 12 h.
11 am (Monday)
- Infect 0.6 MOI live or HI IAV/X31 to 500.000 MDDCs per tube, 8 h.
- Thaw sorted T cells. Wash 2x in R10. Let them rest in the incubator.
- Count cells: 24×10^6 cells
3 pm (Monday)
- Infect 0.6 MOI live or HI IAV/X31 to 500.000 MDDCs per tube, 4 h.

CFSE labelling of T cells
- Resuspend cells to 2×10^6 cells/ml in PBS.
- Thaw fresh aliquot of CFSE from -20ºC (10mM).
- Add 10µl of stock to 10mL of PBS (10µM).
- Prepare 2x CFSE dilution by making 1:10 dilution (1µM).
- Combine cells and 1µM CFSE at a 1:1 ratio, mix quickly and incubate at 37ºC in waterbath for 10 minutes. (0.5µM).
- Add 1 mL of FCS and top up with R10. Wash 2x in R10.
- Re-count T cells. 13×10^6 cells

Setting up co-culture
7 pm:
- Seed 1×10^6 T cells in 1 mL of R10 per FACS tube.
- Wash in PBS and fix 100.000 MDDCs from all conditions in 1% PFA. Wash 3x in R10. Re-count MDDCs.
- Add MDDCs at 1:30 ratio to the T cells (30.000 MDDCs per 1.000.000 cells).
- Add 10µg/mL SEB in the positive control (tube 14 of T cells).
- Seed remaining DCs on coverslips for IF.

160711
- After 7 days incubation, stain tubes for T cell proliferation and analyse by FACS.

3b   **Week 27**

Presentation of IAV to memory CD4 and CD8 T cells by DCs

MDDCs

No.	Cell no.	Treatment	Start Time
1.	500.000	Uninfected	-
2.	500.000	4h IAV	3 pm
3.	500.000	4h HI IAV	3 pm
4.	500.000	4h LPS	3 pm
5.	500.000	8h IAV	11 am
6.	500.000	8h HI IAV	11 am
7.	500.000	8h LPS	11 am
8	500.000	12h IAV	7 am
9.	500.000	12h HI IAV	7 am
10.	500.000	12h LPS	7 am
11.	500.000	24h IAV	7 pm (-1)
12.	500.000	24h HI IAV	7 pm (-1)
13.	500.000	24h LPS	7 pm (-1)

T cells (CFSE-labeled T cells)

No.	T cell no.	DC no.	DC Treatment	Fixed DCs?
1.	1.000.000	30.000	Uninfected	No
2.	1.000.000	30.000	4h IAV	No
3.	1.000.000	30.000	4h HI IAV	No
4.	1.000.000	30.000	Uninfected	Yes
5.	1.000.000	30.000	4h IAV	Yes
6.	1.000.000	30.000	4h HI IAV	Yes
7.	1.000.000	30.000	8h IAV	Yes
8.	1.000.000	30.000	8h HI IAV	Yes
9.	1.000.000	30.000	12h IAV	Yes
10.	1.000.000	30.000	12h HI IAV	Yes
11.	1.000.000	30.000	24h IAV	Yes
12.	1.000.000	30.000	24h HI IAV	Yes
13.	1.000.000	0	-	-
14.	1.000.000	30.000	SEB	No

3c   **Week 28**

Presentation of IAV to memory CD4 and CD8 T cells by DCs

- Wash in 3ml PBS 2% FCS 5min 1500rpm.
- Add 5 µl Live/Dead Blue (1:40 in PBS) for 5 min at 4°C.
- No wash. Add 5 µl FcR block (Miltenyi) to all tubes, 5 min at 4°C
- No wash. Add surface Abs for 15 min at 4°C.
- Wash in 3ml PBS (to remove FCS) 5min 1500rpm.
- Fix in 50µl 1% PFA.

Fortessa T cell proliferation panel:

Laser	Fluorochromes	Marker	Per Test (µl)	Total (n=15)
Blue 488	PerCP Cy5.5	CD8	2	
Blue 488	FITC	CFSE		
Green 561	PE Cy7	CD45 RA	3	
Green 561	PE	CD4	5	
Red 640	APC Cy7	CD3	3	
Violet 405	V450	CCR7	2	
UV	Indo I Violet	Live/dead blue	5 (1:40)	

Compensation Controls

- For live/dead comp, kill cells with ethanol for 5 min. Wash out ethanol with FACS wash. Add 5 µl Blue Live/Dead (1:40 in PBS) for 5 min at 4°C.
- For other comps, add 50 µl positive beads to tubes. Add ab for 15 min at 4ºC. Fix with 50 µl 2% PFA. No wash.

Laser	Fluorochromes	Marker	Per Test (µl)
Blue 488	PerCP Cy5.5	CD8	2
Blue 488	FITC	CD4	3
Green 561	PE Cy7	CD45 RA	1
Green 561	PE	CD8	2
Red 640	APC Cy7	CD20	4
Violet 405	V450	CCR7	2
UV	Indo I Violet	Live/dead blue	5 (1:40)

4

Week 27
160707

IF Staining of MDDCs infected with IAV/X31

1. Wash coverslip with PBS twice.
2. Wash coverslip with PBS twice and p.s. twice.
3. Permeabilize (100 uL p.s./coverslip) for 10 min RT.
4. Wash coverslip with PBS twice.
5. Block with b.s. for 1 h.
6. Add primary Ab (diluted in p.s.), incubate 1 h RT.
      Rabbit PINDA (1:2000)
      Mouse FK21 (1:100)
7. Wash 4 times with b.s..
8. Add secondary Ab (diluted in p.s.), incubate 1 h RT.
      Anti-rabbit 488 (1:500)
      Anti-mouse 555 (1:1000)
9. Wash 4 times with b.s..
10. Add 100μl 10% NMS (normal mouse serum in p.s.) to block for 15 min RT.
11. Add 100μl HLA-DR 647 (1:50) (diluted in p.s.), incubate 1 h RT.
12. Wash 4 times with b.s..
13. Wash with PBS twice.
14. Mount coverslips on glass slides; put drop of mounting solution on the glass slide. Take the coverslip with forceps, tilt to remove PBS. Dip coverslip in water to remove salt from the PBS. Touch filter paper to dry (only the edge) and then flip upside down on one side of the drop of mounting solution. Leave at RT for 20 minutes. Store in fridge.

Ab	488	555	647
1°	PINDA (1:2000)	Ms FK2 (1:100)	HLA-DR 647 (1:50)
2°	Anti-rabbit 488 (1:500)	Anti-Mouse AF 555 (1:1000)	

Blocking solution (b.s.)
PBS + 1% goat serum

Permeabilisation solution (p.s.) (0.1%)
50mL blocking solution + 50 μL Triton X-100.
Ensure that blocking solution is at RT prior to adding Triton X-100.

Glass slides:

160707 MDDC 268 PINDA 488 FK2 555 HLA-DR 647	Uninf	4h IAV	4h HI IAV	

160707 MDDC 268 PINDA 488 FK2 555 HLA-DR 647		24h IAV	24h HI IAV	

B Raw data from Flow Cytometry

The principle of CFSE staining is that with proliferation the intensity of the dye will decrease. The numbers represent percentage T-cells negative to the CFSE dye, meaning they are a result of repeated proliferation.
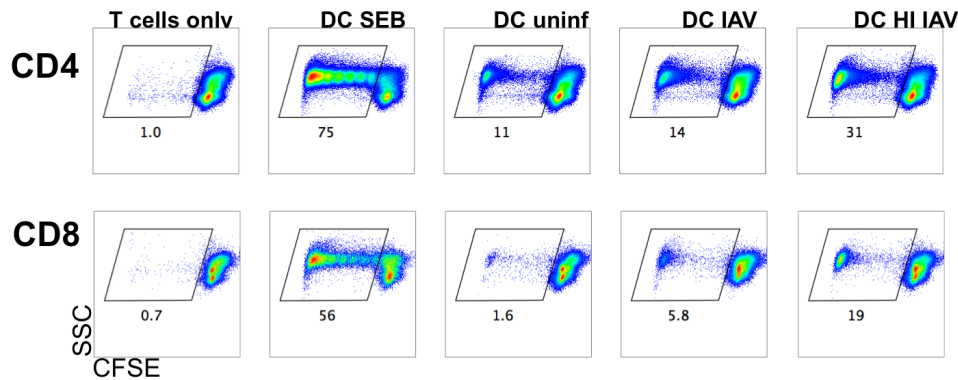

Figure 14: Measurement of intensity of CFSE dye in CD4- and CD8-T-cells and the T-cell proliferation due to antigen presentation by unfixed DCs

Figure 14 shows the percentage of the T-cells expressing lower levels of CFSE. IAV infected, SEB infected, uninfected and HI-IAV infected DCs and no DCs are co-cultured with T-cells resulting in different responses. CD4- and CD8-T-cell responses is separate.
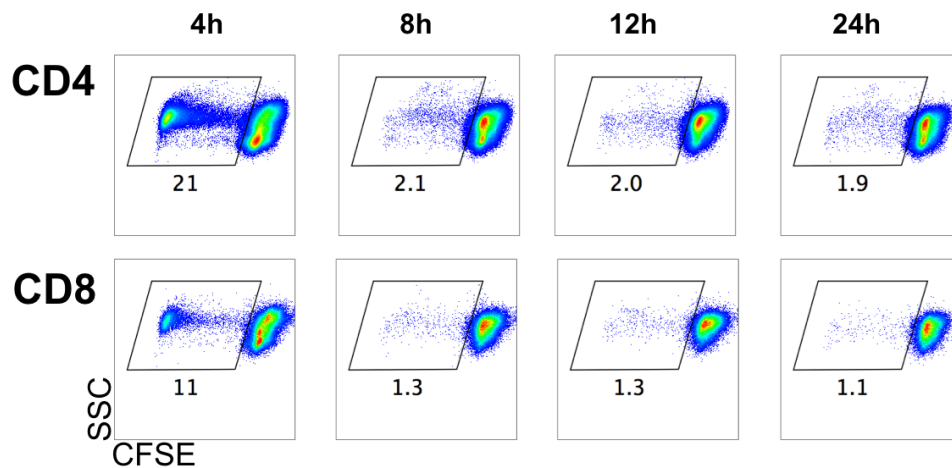

Figure 15: Measurement of intensity of CFSE dye in CD4- and CD8-T-cells and T-cell proliferation due to antigen presentation by fixed DCs infected by IAV

In Figure 15 and 16 the percentage of the T-cells expressing lower levels of CFSE is presented. CD8- and CD4-T-cells are co-cultured with DCs infected with HI-IAV (Figure
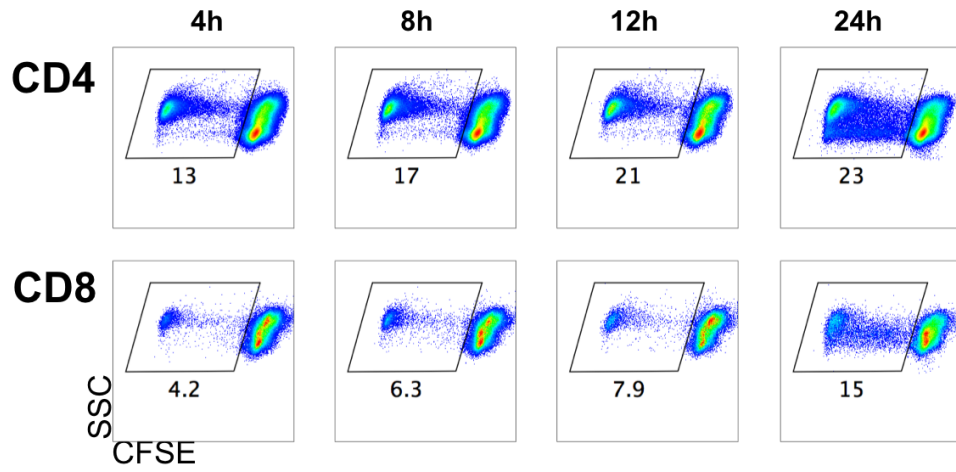
Figure 16: Mesurement of intensity of CFSE dye in CD4- and CD8-T-cells and T-cell proliferation due to antigen presentation by fixed DCs infected by HI-IAV

16) or IAV (Figure 15) fixated at 4 hours, 8 hours, 12 hours and 24 hours after infection before the co-culturing.