Cross talk between dendritic and natural killer cells in the presence of vaccine agent against cervical cancer

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Cross talk between dendritic and natural killer cells in the presence of vaccine agent against cervical cancer

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PREFACE

Graduating as a master in Clinical Molecular Life Sciences is not something you can do on your own. That's why this thesis is the perfect moment to look back and speak out my appreciation.

First of all I would like to thank Prof. J. Boniver and Prof. P. Delvenne for giving me the opportunity to do my senior internship in their laboratory.

I would like to thank my promotor, dr. N. Jacobs for welcoming me into the laboratory, giving me the opportunity to work on this project and for critically reading my thesis. I also want to thank my co-promotor dr. A. Reschner, for her trust, guidance and support and my intern promotor, Prof. J.M. Rigo, for his involvement.

Special thanks to Virginie Renoux and Estelle Dortu, for their help and teaching me their skills.

I also want to thank the other colleagues of the laboratory for the nice moments we shared.

I would like to say thanks to my parents and friends. Mama and papa, thank you for giving me the opportunity to study and always being there for me. Although difficult moments occurred, you kept believing in me. Finally I want to thank Michael, for just being there for me, in good and stressful times!

Thank you all for your support!

Inge

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LIST OF ABBREVIATIONS

APC	Antigen Presenting Cells
APC	Allophycocyanin
CD	Cluster of differentiation
CIN	Cervical Intraepithelial Neoplasias
CLR	C-type Lectin Receptors
CTL	Cytotoxic T cells
DC	Dendritic Cells
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked Immunosorbent Assay
FACS	Fluorescence Activated Cell Sorting
FasL	Fas Ligand
FITC	Fluorescein Isothiocyanate
FSC	Forward Scatter
GM-CSF	Granulocyte Macrophage-Colony Stimulating Factor
HLA-DR	Human Leucocyte Antigen-DR
HPV	Human Papillomavirus
HR-HPV	High-Risk Type of Human Papillomavirus
HRP	Horse Radish Peroxidase
IFN	Interferon
IL	Interleukin
LPS	Lipopolysaccharides
mAb	Monoclonal Antibodies
mDC	Myeloid Dendritic Cells
MHC	Major Histocompatibility Complex
MICA/B	Major Histocompatibility Complex class I-related
NK	Natural Killer cells
NKT	Natural Killer T- cells
NLR	Nucleotide Oligomerization Domain-like Receptors
NOD	Nucleotide Oligomerization Domain

PBMC	Peripheral Blood Mononuclear Cells
pDC	Plasmacytoid Dendritic Cells
PE	Phycoerythrin
PerCp	Peridinine Chlorophyll Protein
PMT	Photomultiplier Tubes
PRR	Pattern Recognition Receptors
RAET	Retinoic Acid Early Transcript
RB	Retinoblastoma
SCC	Squamous Cell Carcinoma
SIL	Squamous Intraepithelial Lesions
SSC	Side Scatter
Th1	T helper 1
Th2	T helper 2
TMB	Tetramethylbenzidine
TNF	Tumour Necrosis Factor
TLR	Toll-like Receptors
TRAIL	TNF-related Apoptosis-Inducing Ligand
VLP	Virus Like Particles
WT	Wild Type

ABSTRACT

Cervical cancer, the second most frequent gynecological malignancy in the world, is caused by infection with high-risk human papillomaviruses (HPV). Major advances in preventive methods for cervical cancer have been achieved with the recently licensed HPV prophylactic vaccines based on HPV virus like particles (VLP). These vaccines proved highly efficient to protect against HPV-16 and HPV-18 infection, but not against established infection. Previous studies have shown that HPV-VLP were able to activate dendritic cells (DC) as demonstrated by a partially matured phenotype and cytokine production. Studies of complex bidirectional cross talk between natural killer (NK) cells and DC underscored the influence of NK cells on activation and maturation of DC. NK cell activation in vaccination protocols has been rarely studied; we sought to study the effect of HPV-VLP on these cells in an autologous setting.

We confirmed previous studies and when we co-cultured VLP activated DC and NK cells, we observed an increase in cell surface expression of CD69. The up-regulation of activation markers on NK cells was also accompanied by increased IFN-γ secretion in the presence of VLP. Preliminary results also showed that the activation of NK cells for CD69 is cytokine dependent and for HLA-DR cell-cell contact dependent. Interestingly, NK cells seemed to further activate DC in the presence of VLP as shown by up-regulation of HLA-DR, CD86 and CD80 on DC. Moreover we found that NK cells synergise with VLP in modulating DC cytokine secretion, since the presence of NK cells lead to further increase in IL12p70 production by DC in the presence of VLP. Furthermore, no modulation was observed for the anti inflammatory cytokine IL-10.

Our results suggest that NK could play a role in the activation of DC induced by HPV-VLP during the vaccination against cervical cancer and DC in presence of HPV-VLP stimulate NK cells which could be potent effector cells against HPV-infected cells.

1. INTRODUCTION

1.1 Cervical cancer

Despite improvements in early diagnosis and treatment, cervical cancer continues to be worldwide the second most common cause of death by cancer that affects women [1]. It is an important health problem in which the etiological agents are some types of human Papillomaviruses (HPV) [2].

1.1.1 Human Papillomavirus

HPV belongs to the Papillomaviruses, which are nonenveloped, double-stranded DNA viruses. The genome of HPV is small, the size is close to 8kb (figure 1). These genes code for 6 non-structural early proteins (E1, E2, E4, E5, E6 and E7) which are important for the virus life cycle and for 2 structural or late proteins (L1 and L2) which form the viral capsid [3].

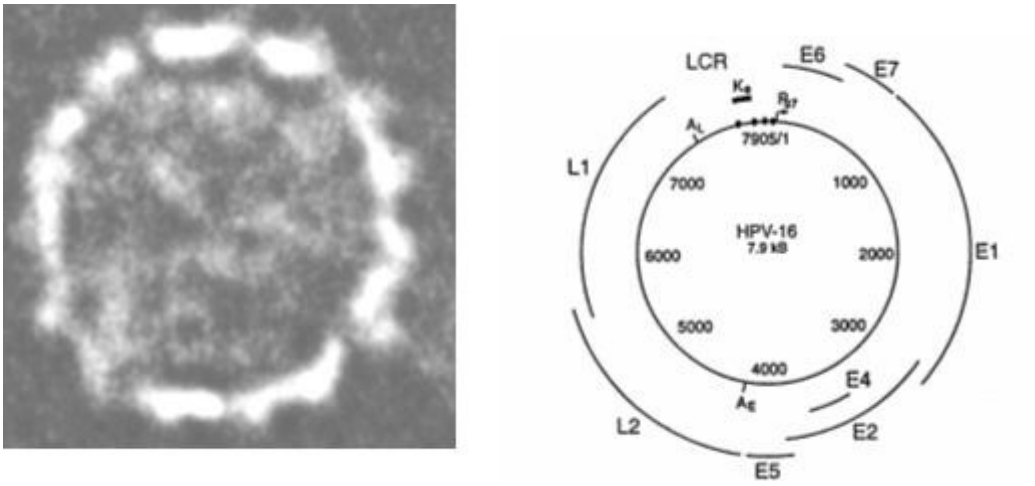

Figure 1: Schematic representation of the HPV genome. E1 and E2 are involved in the replication of the viral DNA. E4 is involved in the transcription of the viral DNA. E5 is involved in the growth stimulation. E6 and E7 are the oncogenes of the virus. L1 and L2 form the viral capsid. (derived from Verschraegen C. F. et al., [4])

More then 100 HPV types are characterised based on the isolation of complete genomes [5]. These HPV types can be divided into 2 groups based on their potential to cause malignancies. The high-risk types are associated with human tumours and the low-risk types with benign hyperplastic lesions. Fifteen different types of HPV are classified as high-risk types of human

papillomavirus (hr-HPV) and three types were considered as probably high risk [6]. The most common identified hr-HPV are HPV16 and HPV18 [6]. These types possess genes (E6 and E7) that, after integration into the cellular genome, encode proteins that inactivate or block tumour suppressor genes like p53 and RB1, respectively, in target epithelial cells and activate cell-cycle related genes such as cyclin E, which causes an uncontrolled cellular proliferation [7] [8]. HPV can be transmitted during sexual activity. It infects the stratified squamous epithelia of the skin and mucous membranes, where they can cause benign lesions (nongenital and anogenital skin warts, oral and laryngeal papillomas, and anogenital mucosal condylomata) and some of these have the potential to progress to an invasive cancer [3].

1.1.2 Pathology of cervical cancer

Cervical cancer is classified into two main types, cervical squamous cell carcinoma (SCC) and cervical adenocarcinoma [9]. Hr-HPV are detected in 99, 7% of the cervical SCC and in 94-100% of the cervical adenocarcinomas. Cervical SCC develops from already existing non-invasive squamous precursor lesions, also called cervical intraepithelial neoplasias (CIN) or squamous intraepithelial lesions (SIL). CIN is identified histologically on the basis of atypia of epithelial cells which extends from the lower parabasal layers of the squamous epithelium up to the whole thickness of the epithelium. There are three distinct CIN classifications known to categorise abnormal cell growth: CIN 1, CIN 2 and CIN 3. The progress from CIN to invasive cancer is illustrated in figure 2 however not all cases of CIN progress to an invasive cancer. First the human papillomavirus infects the basal keratinocytes, which causes an expression of the early HPV genes E1, E2, E5, E6 and E7 and replication of the viral DNA. In the upper layers of epithelium the viral genome is replicated further, and there is expression of E4, L1 and L2. The progression to invasive cancer is associated with loss of E2 due to the integration of the HPV genome into the host chromosomes and the up-regulation of E6 and E7. The time between the acquisition of the HPV infection and the malignant progression usually takes at least 10 years and is frequently longer [10] [11].

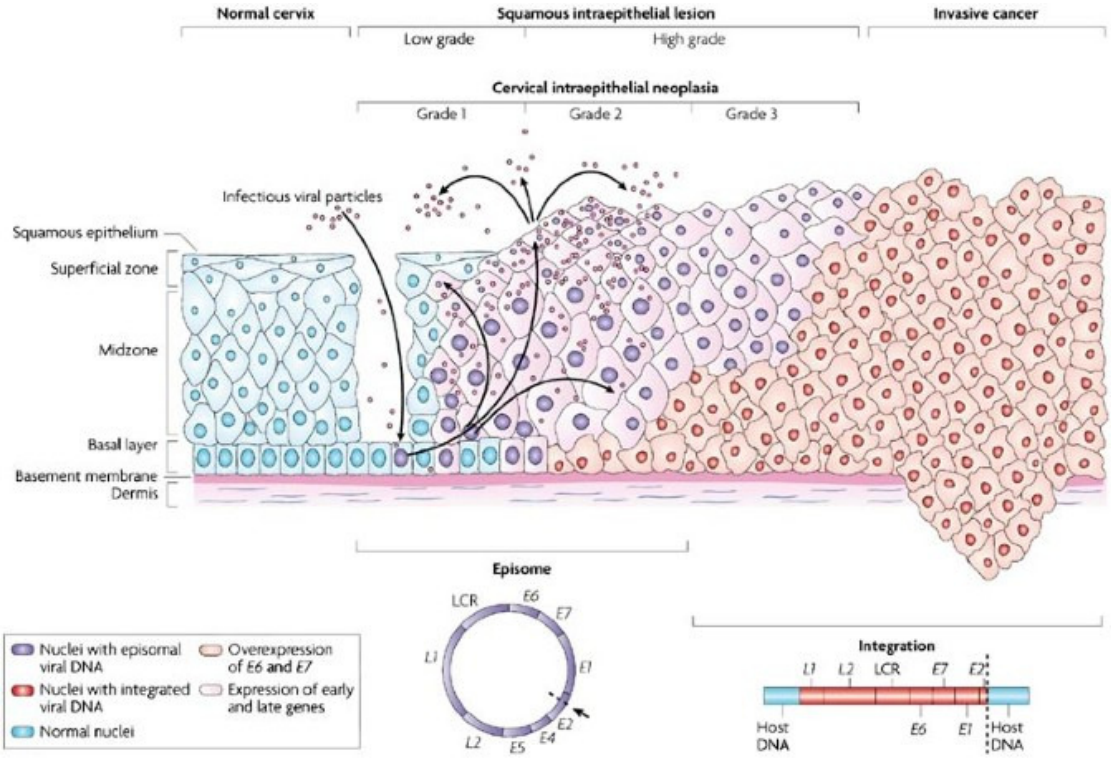

Figure 2: Schematic figure to show the pathology of cervical cancer following human papillomavirus (HPV) infection. HPV infects the basal keratinocytes which causes the expression of E1, E2, E5, E6 and E7 and replication of the viral DNA. In the upper layers of epithelium the viral genome is replicated further, and the late genes L1 and L2, and E4 are expressed. The progression of untreated lesions to microinvasive and invasive cancer is associated with the integration of the HPV genome into the host chromosomes, with associated loss or disruption of E2, and subsequent up-regulation of E6 and E7 oncogene expression. (derived from Woodman C.B. et al., [11])

1.1.3 Vaccines against cervical cancer

Recently, two manufacturers have developed prophylactic HPV vaccines. These vaccines are composed of non-infectious, recombinant HPV virus-like particles (VLP, figure 3). VLP are geometric icosahedral structures which are obtained through self-assembly of the structural HPV protein L1 which contains the immunodominant neutralisation epitopes of the virus. They generate a strong humoral immune response [12]. These prophylactic vaccines contain no live, attenuated or killed virus [13].

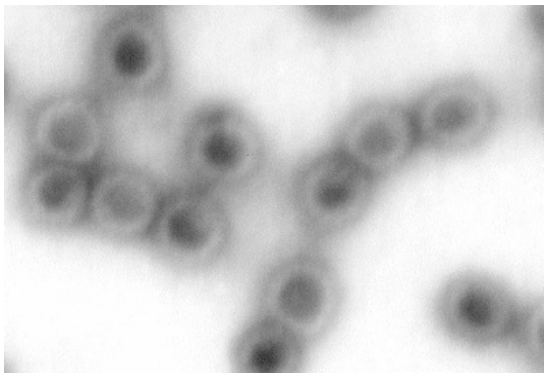

Figure 3: Electron micrograph of HPV virus-like particles

Gardasil (Whitehouse Station, NJ) is one of the two prophylactic HPV vaccines. It includes four vaccine immunogens for HPV types 6, 11, 16 and 18. HPV types 6 and 11 are low-risk virus types that cause approximately 90% of genital warts. The vaccine is produced in yeast through recombinant methods and is adjuvanted with alum. It was approved in June 2006 by the US FDA [13-15].

The other vaccine on the market, is called Cervarix (GlaxoSmithKline Biologicals, Rixensart, Belgium). This is a divalent HPV VLP vaccine which contains immunogens for HPV types 16 and 18. It is produced in insect cells via recombinant baculovirus [16, 17].

These two HPV vaccines potentially provide protection against the two types of HPV that cause approximately 70% of the invasive cervical cancers world-wide: HPV types 16 and 18. But there are several issues that still need to be addressed. One of them is that the duration of the protection is unknown. A second issue is that VLP vaccines are relatively expensive and that vaccine delivery in the developing world will be difficult. Another issue is that the vaccines will only protect against the hr-HPV types that the vaccine targets (HPV types 16 and 18) so the women must continue to undergo Pap smear screening, because they could still be infected by hr-HPV types which are not included in these vaccines. A last issue is that these prophylactic vaccines provide limited benefits to women which are already infected with hr-HPV [18].

1.2 Immune system

The immune response is divided into two major components: an innate component that functions as a first-line of defence and an adaptive component that takes longer to mobilise but confers specificity and exhibits memory. Normally, infections are self-limited, because the host mounts a successful immune response. HPV infections are to slow to induce measurable immune responses and to slow to clear. This suggests that HPV may have developed methods to evade host immune mechanisms. The primary mechanism is via avoidance of antigen presentation. The absence of cell lysis and systemic viremia minimises the availability of antigens for presentation and ensures that only few pro-inflammatory signals are given in the course of infection to invoke adaptive immune responses. Capsid protein expression is only limited to superficial epithelial cells which likely reduces the presentation of capsid proteins to the immune system [19]. The E6 and E7 non-structural proteins of HPV have pleiotropic activity on the biology of infected epithelial cells and delay cell differentiation. E6 can selectively inhibit the interferon response, which is one of the first lines of host defence against viral infection. E7 represses the MHC class I heavy chain gene promoter which may lead to a loss of recognition of virus infected cells by MHC class I-restricted cytotoxic T cells (CTL) [20, 21].
Research into anti-tumour and anti-viral immunity is generally focused on the adaptive immune response. However more and more evidences show the importance of the innate immune response in anti-tumour and anti-viral immunity [22, 23]. Dendritic cells (DC) and natural killer (NK) cells represent two specialised cell types of this immune response.

1.2.1 Dendritic cells

DC represent a small population of bone marrow derived leukocytes which act as biological sensors. DC capture microbes, present their antigens and provide signals necessary for T-cell expansion and differentiation. Immature DC recognise microbial components through pattern recognition receptors (PRR). Examples of these PRR are Toll-like receptors (TLR), cell surface C-type lectin receptors (CLR) and intracytoplasmic nucleotide oligomerization domain (NOD)-like receptors (NLR) [24]. Immature DC up-regulate costimulatory molecules (CD40, CD80 and CD86), when they recognise microbes or as a response to inflammatory cytokines which are secreted by cells in the tissue microenvironment. After the up-regulation of costimulatory molecules, they migrate to secondary lymphoid organs [25], where they

activate antigen-specific T cells [26] [27]. DC are important in the humoral immunity because they have the capacity to directly activate B cells [28]. They can activate innate immune cells such as natural killer (NK) cells and natural killer T (NKT) cells [29, 30].

In humans two different subtypes of DC are described. These subtypes are defined as myeloid DC (mDC) and plasmacytoid DC (pDC) [31]. The mDC express CD11c and other myeloid markers including CD1a, HLA-DR, CD11b, CD33 and $CD14^{low}$. They also possess the Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF) receptor. For growth and survival they are dependent on GM-CSF. These DC secrete IL-12 when they are stimulated by a CD40 ligand or when they undergo maturation by TLR. This cytokine is important for the induction of cytotoxicity and Th1-type secretions by T lymphocytes. Matured mDC are characterised by an increase of the markers CD80, HLA-DR, CD86 and CD83 [32]. The pDC lack myeloid markers, they are CD4+CD45RA+CD123+CD11c- and for survival they depend on IL-3 and IFN-γ. pDC are potent producers of IFN-α when they are stimulated by viruses and they can also process and present virus antigens to CD4 and CD8 T cells [33].

1.2.2 Natural killer cells

NK cells are specialised innate lymphocytes which are capable of responding to virus-infected and tumour cells [34]. Originally they were identified by their ability to spontaneously mediate lysis of certain susceptible tumour cell lines and their large granular lymphocyte morphology. They were characterised as non-T cells which lack the expression of T cell antigen receptors and the CD3 complex. NK cells represent approximately 10% of all peripheral blood lymphocytes and express CD56, CD16 and NKp46 [35, 36]. According to the cell surface density of CD56 in humans two different subtypes of NK cells were described. The majority (approximately 90%) are $CD56^{dim}$ cells and the minority (approximately 10%) are $CD56^{bright}$ cells. The $CD56^{bright}$ NK cells produce in response to monokine stimulation greater levels of interferon (IFN)-γ, tumor necrosis factor (TNF)-β, GM-CSF, IL-10 and IL-13 in comparison with the $CD56^{dim}$ NK cells [37].

The function of NK cells is tightly regulated by a balance between positive and negative signals provided by the cell surface receptors [38]. In table 1 all the surface receptors present on human NK cells are summarised.

Table 1: Summary of all the surface receptors with their ligands and function which are present on human NK cells.

	Ligands	Function
Kir	HLA-A, B, C allotypes	Activation and inhibition
NKG2A-C/CD94	HLA-E	Inhibition
NKG2D	MICA/B, RAET	Activation
NCR (NKp30,44)	Virus?	Activation
CD16	Immune complexes	Activation
CD161	clr-g, clr-b	Activation and inhibition
CD27	CD70	Activation

HLA: human leucocyte antigen; MICA/B : major histocompatibility complex class I-related; NK : natural killer; MHC: major histocompatibility complex; RAET: retinoic acid early transcript.

Activation of NK cells requires the action of pro-inflammatory cytokines (IL-12, IL-15, IL-18, IL-21, IFN-αβ and IFN-γ) in combination with differential engagement of cell surface receptors [39-43]. Inhibition occurs by receptors that recognise MHC class I molecules. Healthy cells which express normal levels of MHC class I are generally protected from NK cell-mediated lysis (figure 4).

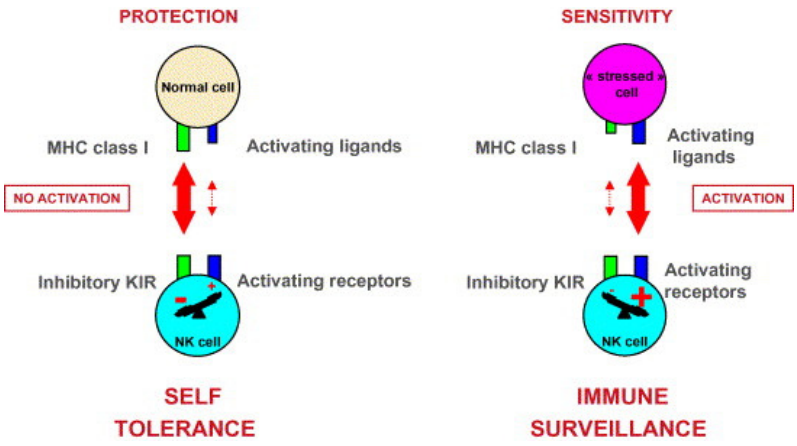

Figure 4: Schematic representation of the cytotoxic activity of NK cells which is dependent on the balance between inhibitory and activating signals. Cells which express normal levels of MHC class I are protected from NK cell-mediated lysis. (derived from Vivier et al., [35])

The effector functions of NK cells, such as cytotoxicity and the production of a variety of cytokines, are pathophysiological important. NK cells can kill target cells by two major mechanisms that require direct contact between NK cells and target cells. The first mechanism is via perforin/granzyme granule-mediated exocytosis. In NK cells, there are

cytotoxic granules which contain perforin, granzymes and granulysin. When the NK cells become activated, they release the cytotoxic granule contents into the intercellular space between the NK cell and the target cell. The second mechanism is via signaling through the TNF death receptor family members. TNF-related apoptosis-inducing ligand (TRAIL) and Fas ligand (FasL) are expressed on NK cells. They are important mediators of apoptosis that shape and regulate the immune system. The expression of TRAIL can be induced by IFN-γ [44]. This induction causes an increase of the cytotoxic capacity of NK cells. It is likely that TRAIL plays a role in the antiviral activities of NK cells following the release of IFN-αβ by infected cells [45]. The expression of FasL contributes to the suppression of tumour growth [46]. Many tumour cells normally don't express Fas but NK cells induce directly the expression of Fas on cancer cells via IFN-γ secretion and kill them in a Fas-dependent manner [47].

1.2.3 The interaction between dendritic cells and natural killer cells during the activation of the immune response

Previous work already showed that there are interactions between DC and NK cells. This cross-talk plays a role in the activation of the immune response. The process is illustrated in figure 5.

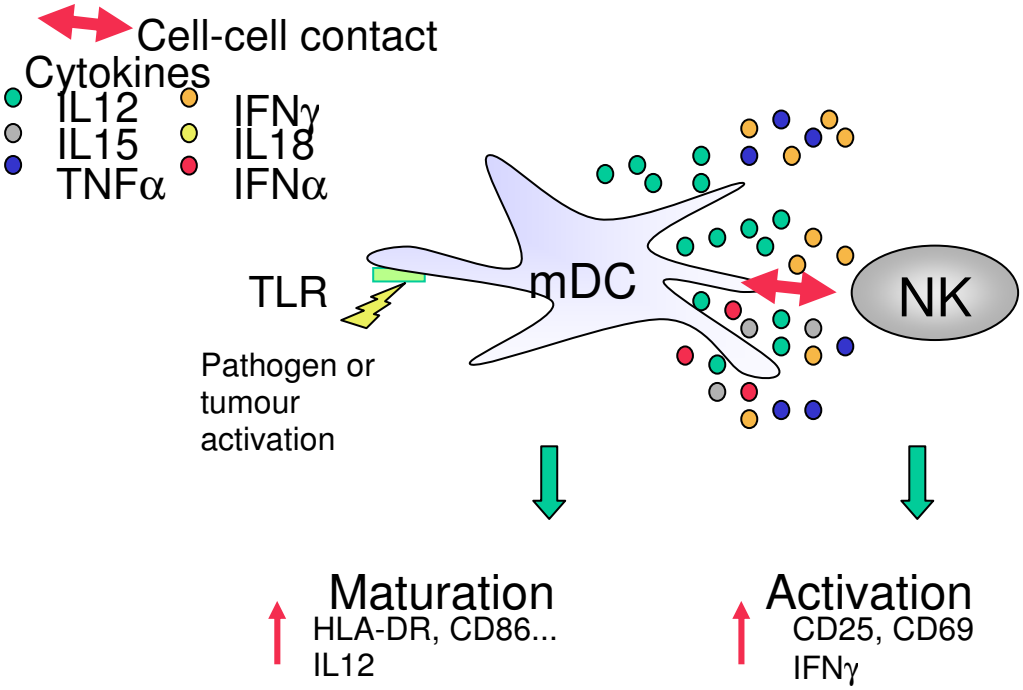

Figure 5: Schematic representation of the cross-talk between DC and NK cells during the activation of the immune response. pDC, plasmacytoid DC; IL, interleukin; IFN, interferon; TNF, tumor necrosis factor; TLR,

Toll-like receptors; mDC, myeloid DC; HLA-DR, human leucocyte antigen-DR. (adapted from Reschner, A. et al., [48]).

Fernandez et al., showed that NK cells can be directly activated by DC in vivo [22]. In vitro activation of resting NK cells induced by DC requires direct cell contact which results in a polarised secretion of pre-assembled stores of IL-12 by DC towards NK cells [49]. Della et al. found that the DC-NK interactions were multi-directional [50]. Besides direct cell-cell contact there are also soluble factors present. Especially IL-2, IL-18 and type I IFN play an important role in this cross-talk. Different subsets of DC seem to activate NK cells at several steps of the immune response. pDC promote the early activity of NK cells followed by mDC. On the other hand, NK cells can also activate DC in vitro via cell-cell contact and the production of TNF-α [51, 52].

1.2.4 The interaction between dendritic cells and natural killer cells during the induction and effector phases of the immune response

The cross-talk between DC and NK cells plays also a role during the induction and effector phase of the immune response. In the presence of tumour cells and certain viruses but absence of inflammation and pathogen-associated molecules, it is important that DC are maturated by innate lymphocytes to induce the early immune response. In lymph nodes, NK cells influence the maturation of DC. This plays an important role in the initiation of specific T cell responses. Mature DC react by activating NK cells to produce IFN-γ, which is necessary for T helper 1 (Th1) polarisation [53, 54]. DC and innate lymphocytes share similar functions like functioning as antigen presenting cells (APC) and cytotoxic properties. NK cells can become potent APC when they are activated. Once they are activated, they can up-regulate MHC class II, CD80 and CD86 molecules and acquire independent unique mechanisms of antigen capturing and presenting. This involves the activating receptors NKp46, NKp30 and NKG2D [55].

1.3 Aim of the study

Recently, two prophylactic VLP-based vaccines against HPV have become available. Clinical trials have demonstrated that vaccination with VLP HPV 16/18-L1 leads to a strong humoral immune response [17]. However several issues have to be addressed. One of them is that these prophylactic vaccines provide limited benefits to women which are already infected with hr-HPV. HPV escapes immune surveillance by targeting the different epithelial DC subsets which are present in the stratified epithelia of the skin and mucosa. DC are important for the activation and modulation of the immune response. Also the interaction between NK cells and DC is important for the induction of the adaptive immune response. For this reason, we investigate the cross talk between DC and NK cells in immune response induced by HPV-VLP vaccination. Results of this study may possibly contribute to an enhanced knowledge about the interaction between DC and NK cells in the combination with the HPV-VLP vaccine. It can enable us to obtain more information to improve the vaccine for the treatment of cervical cancer in humans.

1.3.1 Specific aims

1- To investigate if DC activated by VLP can stimulate NK cells (cell surface expression of activation markers and cytokine production).
2- To investigate if cell-cell contact or soluble factors are involved in the activation of NK cells.
3- To investigate if NK cells in the presence of VLP can activate DC (cell surface expression of maturation markers and cytokine production).

2. MATERIALS AND METHODS

2.1 Cell culture reagents

All cells were cultured into RPMI 1640 medium supplemented with 10% fetal calf serum, 10 000 U/ml penicillin, 10 000 µg/ml streptomycin and 10% non essential aminoacids (Gibco-Invitrogen, Paisley, Great Britain).

2.2 DC generation from PBMC CD14$^+$ cells

Peripheral blood mononuclear cells (PBMC) were derived from buffy coats of healthy donors obtained from the Liège University Blood bank. PBMC were isolated by a Ficoll density gradient centrifugation as followed (figure 6). First the human blood was diluted two times in RPMI 1640 (Gibco-Invitrogen). Thirty ml of the diluted blood was added on top of 15 ml lymphoprep (Axis-Shield, Oslo, Norway). Afterwards there was a centrifugation step (447 g) which took 30 minutes (without brake) to separate the different cell types. After the centrifugation, a white ring was visible at the interface between the lymphoprep and plasma.

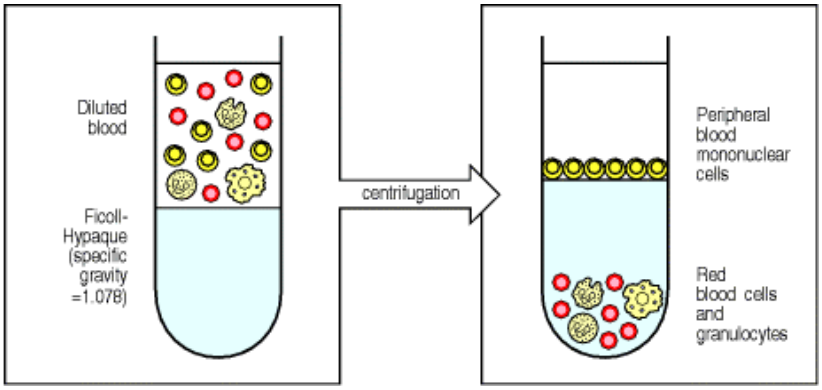

Figure 6: Isolation of peripheral blood mononuclear cells by Ficoll density gradient centrifugation. The erythrocytes and granulocytes have a higher density compared to lymphoprep while leukocytes and platelets have a lower density then lymphoprep. Thus, the two later form a ring at the interface between lymphoprep and plasma. (derived from Janeway CA, et al. [56])

This ring is due to density differences between cell types. The ring was collected and then two times diluted in RPMI 1640. The next step of the isolation was to add 15 ml of the diluted white ring on top of 15 ml optiprep (Gibco-Invitrogen). Afterwards there was a centrifugation step (386 g) which took 15 minutes (without brake). The pellet was washed two times with

RPMI medium (10 min at 447g). The cells where then counted with the help of a counting chamber Thoma. Hundred million of PBMC were used for the isolation of $CD14^+$ cells. The rest of the PBMC were frozen in fetal calf serum supplemented with 10% dimethyl sulfoxide (DMSO) for the isolation of NK cells.

$CD14^+$ cells were isolated by positive selection according to the manufacturer's instructions (Miltenyi Biotec). $CD14^+$ monocytes were differentiated towards DC by culturing during 7 days in RPMI 1640 complete media supplemented with 5ng/ml IL-4 and 100ng/ml GM-CSF. Media was refreshed with cytokines after 3 days. After 7 days of incubation, the maturation of DC was analysed by flow cytometry for the presence of the known surface markers (CD1a, CD86, CD80, CD14 and HLA-DR).

2.3 NK cell isolation

Frozen aliquots of PBMC were thawed and NK cells were negatively selected by using a cocktail of beads (Miltenyi Biotec). The purity of the NK cells was assessed by flow cytometry (CD56, CD16 and CD3).

2.4 DC/NK cell cocultures

DC and NK cells were cultured either alone (0.5 x 10^6 cells/ml) or in the presence of NK cells or DC at a 1:1 ratio for 24 hours. We tested several conditions (figure 7): alone, in the presence of VLP (10µg/ml), wild type (WT, the same volume as VLP) or Lipopolysaccharides (LPS,1µg/ml). Cell culture supernatants were collected and stored at -20 °C until analysed for cytokine production.

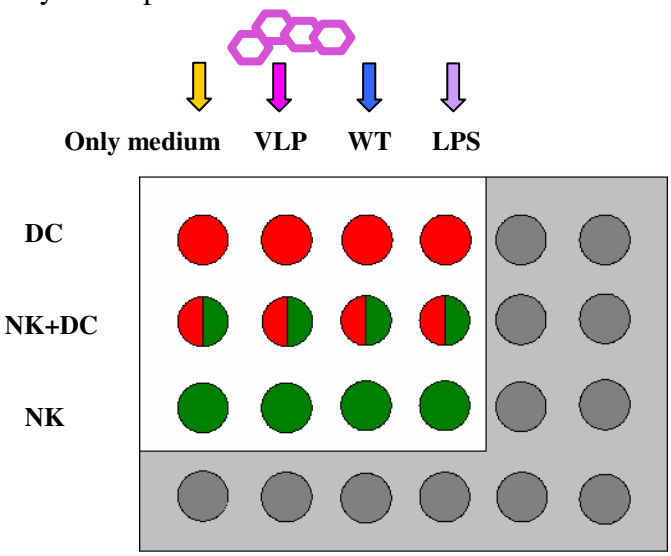

Figure 7: Schematic representation of the DC/NK coculture. In a 24-well plate, we cultured DC and NK cells either alone or in the presence of NK cells or DC at a 1:1 ratio. They were cocultured with or without VLP, wild type (WT) as negative control or Lipopolysaccharides (LPS) as positive control.

In other experiments, we cultured NK cells with supernatants of DC at a 1:1 ratio. The DC were cultured one day in advance without adding anything or in the presence of VLP (10µg/ml), WT (0.1µg/ml) or LPS (1µg/ml).

2.5 Virus-like particles

VLP HPV16 were obtained by infection of Sf9 cells with a recombinant baculovirus encoding the HPV16 L1 protein during 72 hours at 27°C [57]. Cells were harvested by centrifugation, were resuspended in PBS containing 0.5% of NP-40, 10µg/ml Pepstatine A (Sigma) and 1 µg/ml of Leupeptine (Sigma) and were then placed on ice for 30 minutes. Cells lysates were then centrifuged at 12000g for 20 min at 4°C. The nuclear fraction was resuspended in PBS containing 1 µg/ml of both Pepstatine A and Leupeptine and sonicated 3 times for 15 seconds on ice. This fraction was then deposited on preformed CsCl gradient (51%, 45%, 36%, 21% in equal quantity) and centrifuged at equilibrium in a Beckman SW28 rotor (24h, 27 000 rpm, 4°C). L1 positive fractions, analysed by SDS-PAGE gels, were pooled in PBS and centrifuged (3h, 28 000 rpm, 4°C). VLP were resuspended in NaCl 0.15 M. During the first experiments, we used as a negative control WT baculovirus which underwent the same purification process. For latter experiments, we used lysates of insect cells as a negative control. The produced quantity is estimated by a micro BCA protein assay kit (Thermo scientific, Rockford, USA).

2.6 Flow cytometry analyses

Flow cytometry is a powerful method to study and purify cells in immunology and cell biology fields. The instruments used for these analyses are called fluorescence activated cell sorting (FACS) The principle of the FACS is illustrated in figure 8. Individual cells, which are held in a thin stream of fluid, are passed through one or more laser beams and cause light to scatter and fluorescent dyes to emit light at various frequencies. Photomultiplier tubes (PMT) convert light to electrical signals and cell data is collected. Cell sub-populations are identified and sorted at high purity. The data provides information about their size (forward scatter (FSC)), shape, granularity (side scatter (SSC)) and fluorescence properties (fluorescent

labeling). For this study, we analysed our cells with a FACSCanto II (Becton Dickinson, Erembodegem, Belgium) which can analyse 8 colours but with no sorting capacity.

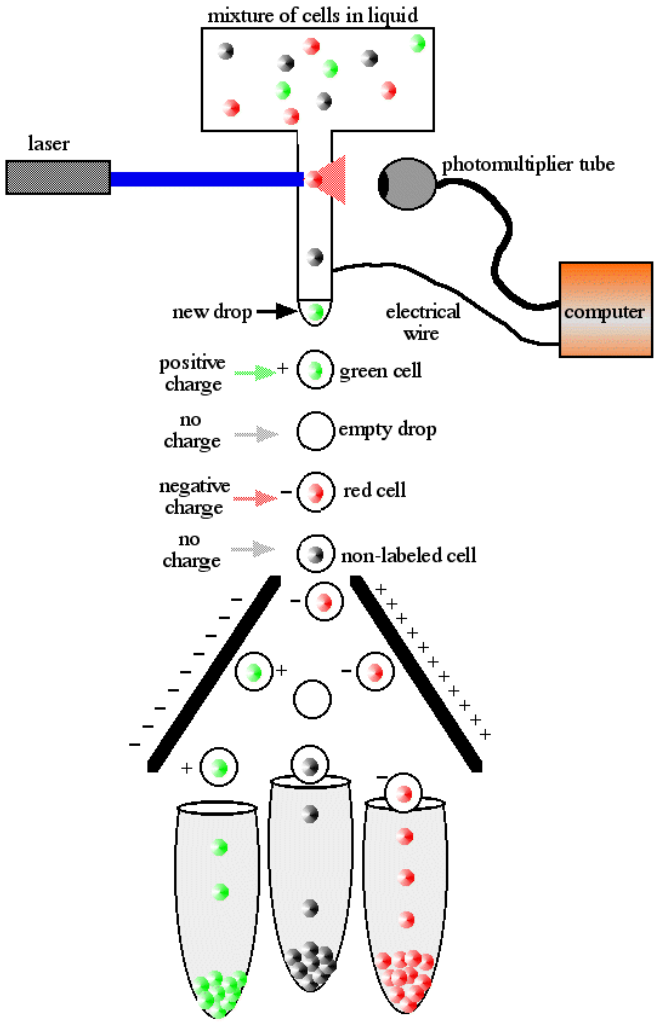

Figure 8: General principle of the FACS. Cells, which are held in a thin stream of fluid, are passed through one or more laser beams and cause light to scatter and fluorescent dyes to emit various frequencies. Photomultiplier tubes convert light to electrical signals and cell data is collected. The data gives information about size, shape, granularity and fluorescence properties.
(derived from http://www.bio.davidson.edu/COURSES/GENOMICS/method/FACS.html

FSC and SSC are used to identify cells. In a peripheral blood sample, lymphocyte, monocyte and granulocyte populations can be defined on the basis of FSC and SSC. They are also used to exclude debris and dead cells.

Fluorescent labelling is used to investigate cell structure and function. Cell autofluorescence is generated by labelling cell structures with fluorescent dyes. FACS collects fluorescence signals in one to several channels corresponding to different laser excitation and fluorescence

emission wavelength. Immunofluorescence, the most widely used application, involves the staining of cells with antibodies conjugated to fluorescent dyes such as fluorescein isothiocyanate (FITC), phycoerythrin (PE), allophycocyanin (APC) and peridinine chlorophyll protein (PerCp). This method is often used to label molecules on the cell surface, but antibodies can also be directed at targets in cytoplasm.

The monoclonal antibodies (mAb) used in our experiments to analyse the DC and NK cell surface markers are summarised in table 2.

Table 2: Summary of the mAb used to analyse the DC and NK cell surface markers.

Antibody	Fluorescent dye	Company	Volume
anti-CD1a	FITC	Dako	5 µl
anti-CD80	PE	BD	
anti-CD86	APC	BD	
anti-HLA-DR	FITC	Dako	
anti-CD83	PE	BD	
anti-CD56	PE	Dako	
anti-CD16	PE	BD	
anti-CD69	FITC	BD	
anti-CD3	PerCp	BD	10 µl

FITC: fluorescein isothiocyanate; PE: phycoerythrin; APC: allophycocyanin; PerCp: peridinine chlorophyll protein; Dako: DakoCytomation, Glostrup, Denmark; BD: Becton Dickinson, Erembodegem, Belgium.

2.7 Detection of cytokines by an enzyme-linked immunosorbent assay (ELISA)

Supernatants from the cocultures were collected and assayed for cytokine ELISA. The following commercially available ELISA kits were used: IL-12p40, IL-12p70, IL-10 and IFN-γ.

2.7.1 ELISA kits

- Human IL-12p40 CytoSet™ (Biosource, Camarillo, USA)

This kit contains:

- capture antibody: working solution 1,0µg/ml

- standard: working solution 2000 pg/ml
- biotin-conjugated antibody: working solution 0,16µg/ml
- streptavidin conjugated Horse Radish Peroxidase (HRP): 2500 times diluted

The detection limit for this kit was 10 pg/ml.

- Human IL-12p70 CytoSet™ (Biosource)

This kit contains:

- capture antibody: working solution 4,0µg/ml
- standard: working solution 2000 pg/ml
- biotin-conjugated antibody: working solution 300ng/ml
- streptavidin-HRP: 200 times diluted

The detection limit for this kit was 50 pg/ml.

- Human IL-10 CytoSet™ (Biosource)

This kit contains:

- capture antibody: working solution 1,0µg/ml
- standard: working solution 2000 pg/ml
- biotin-conjugated antibody: working solution 0,16µg/ml
- streptavidin-HRP: 2500 times diluted

The detection limit for this kit was 10 pg/ml.

- Human IFN-γ CytoSet™ (Biosource)

This kit contains:

- capture antibody: working solution 2,0µg/ml
- standard: working solution 2000 pg/ml
- biotin-conjugated antibody: working solution 0,16µg/ml
- streptavidin-HRP: 2500 times diluted

The detection limit for this kit was 10 pg/ml.

2.7.2 General principle

A 96-well plate was coated with a human capture antibody according to the manufacturer's protocol. After blocking and washing the plate, the standards and samples were added together with a biotin-conjugated antibody (only for IL12p70 there is a washing step between the adding of the samples and the detection antibody). Afterwards there was a washing step to

remove unbound antibodies. The next step was to add streptavidin-HRP. After washing away the excess, tetramethylbenzidine (TMB) was added. The reaction was stopped with 2N H_2SO_4 and the absorption was measured at 450nm. With help of the standard curve, we calculated the concentration of cytokines present in the samples.

2.8 Statistical analysis

All statistical analysis were performed using Graph Pad Prism software. Effects between different coculture conditions were calculated by non-parametric t-tests (Mann-Whitney test). P-values < 0.05 were considered significant.

3. RESULTS AND DISCUSSION

3.1 Phenotype of DC

DC were prepared as described in materials and methods. The quality of differentiated cells was analysed by flow cytometry upon phenotyping with anti-CD1a, anti-CD14, anti-CD80, anti-CD86 and anti-HLA DR antibodies. When we looked at the maturation markers, shown in figure 9, we observed that DC show a shift in CD1a and were CD14 low. They were highly positive for HLA-DR. Furthermore some of these cells showed positivity for another maturation marker CD86, whereas a few were CD80 positive. The purity of DC used in the experiments was 100% for CD11c and 55,5 ± 16% for CD1a (n=14).

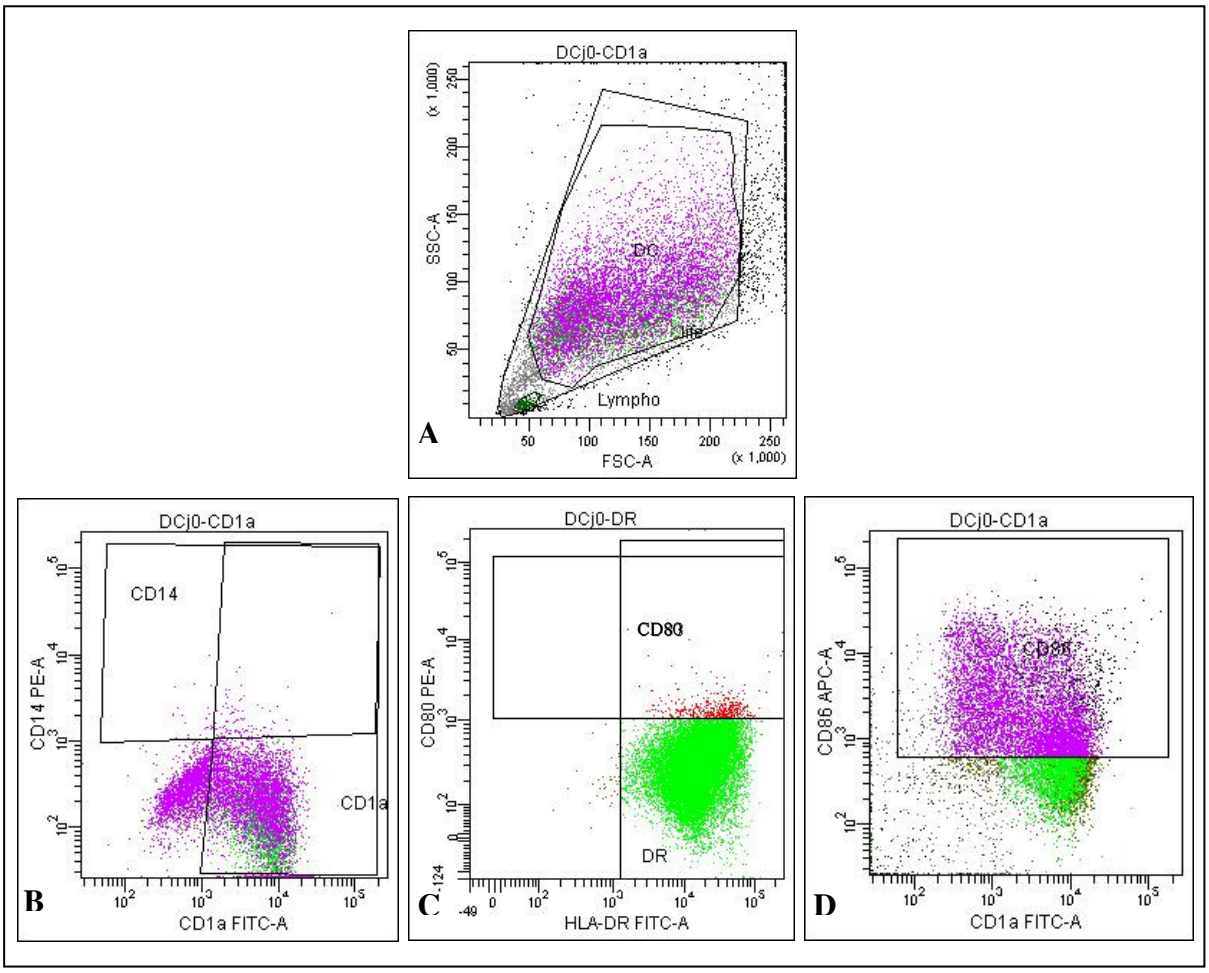

Figure 9: Phenotyping of DC. A: A dot plot of in vitro differentiated DC. X-axis represent forward scatter (FCS) and Y-axis side scatter (SSC). DC gated in A show a shift in CD1a and are low in CD14 (B), are highly positive for HLA-DR and a few are CD80 positive (C). Some are CD86 positive (D).

3.2 Phenotype of NK cells

Autologous NK cells were isolated from frozen PBMC as described in materials and methods. The purity of the NK cells was analysed by flow cytometry upon phenotyping with anti-CD56, anti-CD16 and anti-CD3 antibodies. In figure 10, the phenotype of NK cells before and after sorting is shown. We saw that the percentage of $CD3^+$ cells was very low (<2%), which meant that there was no T lymphocyte cell contamination. When the percentage of $CD3^+$ cells was higher than 2%, we used anti-CD3 PerCp to exclude T lymphocyte cell contamination. Before sorting the purity of NK cells was in average 19,5 ± 8,8%. The purity of NK cells used in the experiments was in average 92,5 ± 4,7%, whereas the amount of contaminant monocytes was in average 5,2 ± 5,7% (n=15).

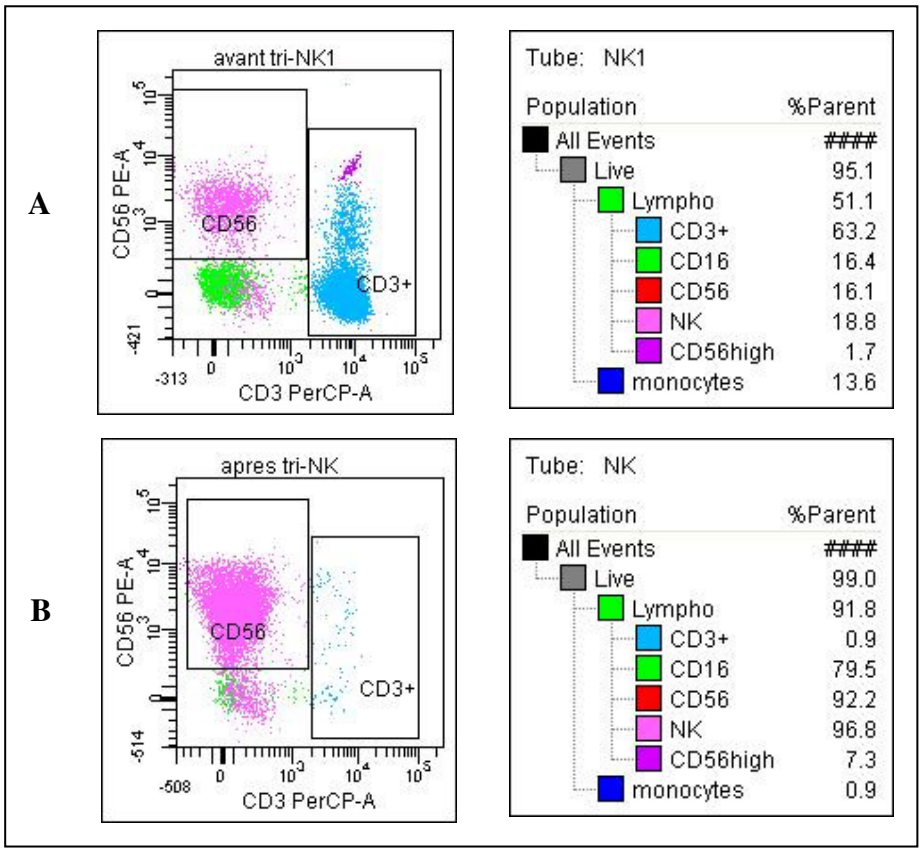

Figure 10: Phenotyping of NK cells before (A) and after sorting (B). NK cells were CD56 high and CD3 negative.

3.3 DC activated by VLP can stimulate activation of NK cell markers

Previous works have shown that HPV-VLP were able to activate DC. They observed a significant up-regulation of the CD80, CD83, CD86 and HLA-DR molecules as well as secretion of IL-12. [58, 59]. Because DC are the most potent inducers of immune responses, these findings could explain the high immunogenicity and efficiency of VLP as vaccines. We confirmed these results for CD80 (see section 3.6 figure 14) and we also studied if DC activated by VLP can stimulate activation of NK cells. This can be very important because the effector functions of NK cells, such as cytotoxicity and the production of a variety of cytokines, have pathophysiological importance. The activation of NK cells was observed by FACS analysis by assessment of the up-regulation of NK surface markers CD69 and HLA-DR. The results are shown in figure 11 and 12, where we looked at the mean fluorescence intensity of CD69 and the percentage of HLA-DR positive cells. We observed an increase of CD69 and HLA-DR cell surface expression on NK cells in the presence of DC and HPV16-VLP. For CD69, there was no significant effect visible between the NK cells alone and the NK cells in combination with VLP. However there was a significant effect observed between NK/DC and NK/DC/VLP ($p<0,03$) as well as between NK/VLP and NK/DC/VLP ($p<0,02$). A significant effect between the positive control conditions NK/LPS and NK/DC/LPS ($p<0,02$) was also found. For HLA-DR, there was a significant effect visible between NK/DC and NK/DC/VLP ($p<0,02$) and also between NK/VLP and NK/DC/VLP ($p<0,001$). During these experiments we also used a negative control (WT Baculovirus) to see if the effect was due to baculovirus/insect cell contaminants. When we looked at the results, we saw that the WT baculovirus showed the same results as NK cells cultured alone or in the presence of DC. So, the significant effect was not due to baculovirus/insect cell contaminants. The obtained results indicate that DC activated by VLP can stimulate the activation of the NK cell markers.

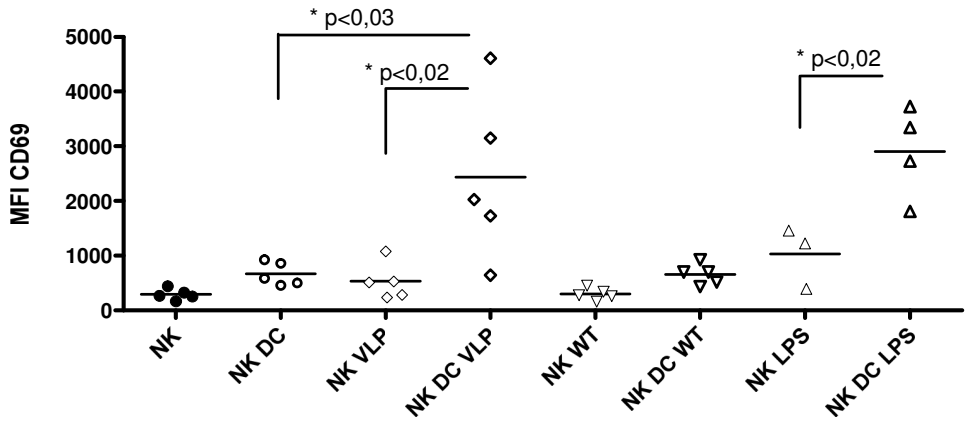

Figure 11: **Mean fluorescence intensity of CD69 on NK cells analysed by FACS.**

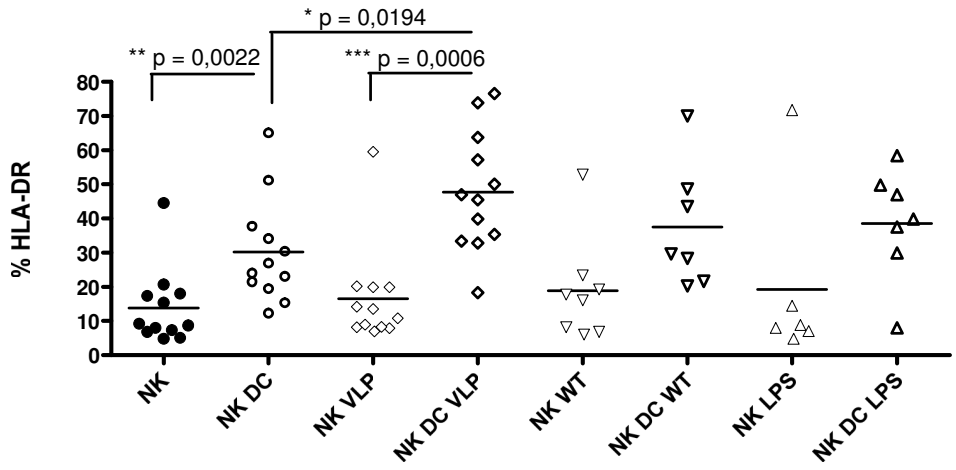

Figure 12: **Percentage of HLA-DR positive cells analysed by FACS**

3.4 NK cell activation due to NK DC cell-cell contact or to soluble factors?

Furthermore we studied if the activation of NK cells was due to cell-cell contact between DC and NK cells or to soluble factors present in the supernatants. We did this as described in materials and methods by adding only the supernatants of DC to the NK cells. These DC were cultured in different conditions (alone, in the presence of VLP, LPS or WT). The activation of NK cells was observed by FACS analysis by investigating if there was an up-regulation of NK surface markers CD69 (A) and HLA-DR (B). The results are shown in figure 13, where we looked at the mean fluorescence intensity of CD69 and the percentage of HLA-DR positive cells. From these preliminary results (n=3) we couldn't conclude anything. But we saw a trend for CD69, there was an increase for NK/DC/VLP compared to NK/DC. This increase is even higher between NK/DC/LPS and NK/DC. This suggest that CD69 up-

regulation could be cytokine-dependent. For HLA-DR, there was no increase visible which could mean that HLA-DR is cell-cell contact dependent. These experiments need to be repeated to become significant values.

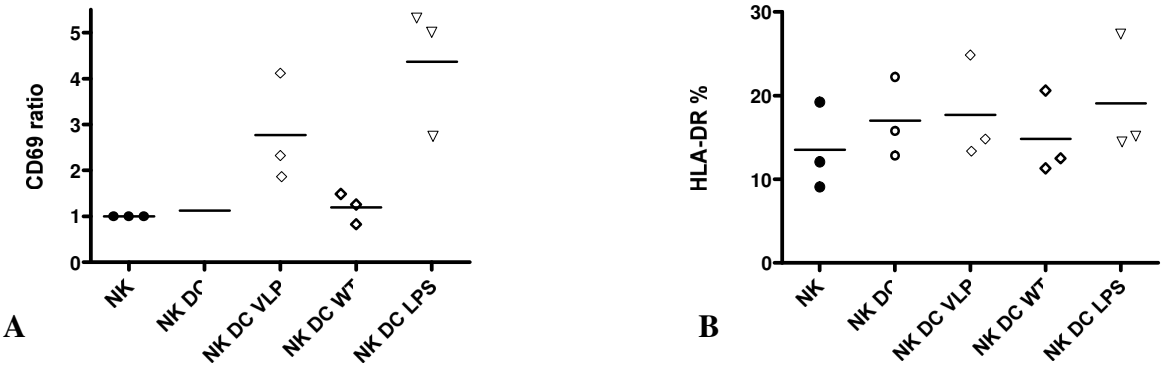

Figure 13: A: Ratio of CD69 on NK cells analysed by FACS. B: Percentage of HLA-DR positive cells analysed by FACS.

3.5 DC activated by VLP can stimulate the production of IFN-γ by NK cells

IFN-γ is a cytokine secreted by NK cells when they become activated. IFN-γ is important because it co-ordinates the link between pathogen recognition by innate immune cells and the induction of specific immunity, by mediating a positive feedback to amplify the Th1 response [60]. Literature already showed that LPS directly triggers IL-12 production upon recognition by DC, which in turn induces IFN-γ secretion by NK cells [61]. We confirmed these results (figure 14) and we further studied if DC activated by VLP can stimulate the production of IFN-γ by NK cells. We did this by performing an IFN-γ ELISA. We observed a significant effect between NK/DC and NK/DC/VLP ($p<0,0001$) and also between NK/DC and NK/DC/LPS ($p<0,001$). A significant effect was as well present between DC and DC/VLP ($p<0,008$). Moreover in these experiments, there was no effect of WT. So the up-regulation of activation markers on NK cells was also accompanied by an increased IFN-γ secretion in the presence of VLP and DC. Therefore we can conclude that NK cells are potent effector cells in the presence of DC and VLP.

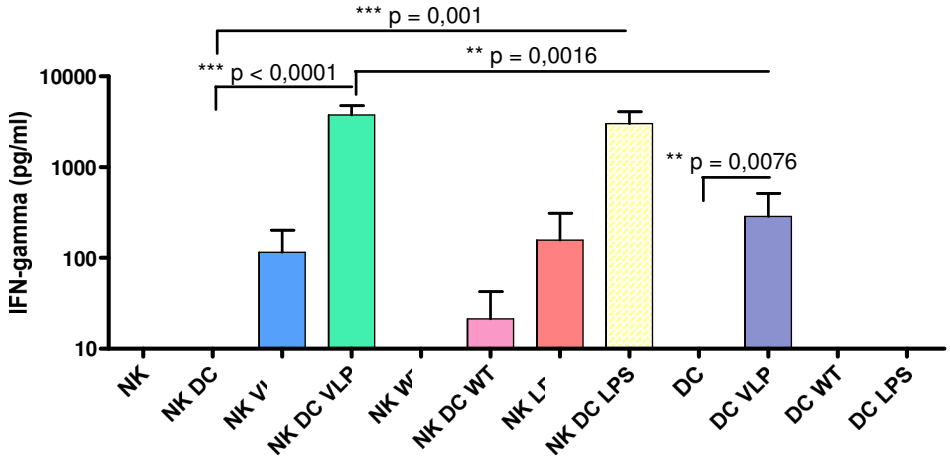

Figure 14: Production of IFN-γ by NK cells.

3.6 NK cells have a synergistic effect with DC in the presence of VLP

Recent studies have demonstrated that DC maturation can be mediated by NK cells [51, 62]. Gerosa et al., showed that in cocultures of NK cells and DC in the presence of DC maturation stimuli not only NK cells become activated but they also observed a much stronger DC maturation than in the absence of NK cells. This is interesting when the absence of pathogen-related molecules or inflammation doesn't lead to DC maturation and effective antigen presentation. We confirmed this result (figure 15) and we studied if NK cells further activate DC in the presence of VLP. The further maturation of DC was observed via FACS analysis by examining the up-regulation of DC surface maturation markers HLA-DR, CD86 and CD80. The results are shown in figure 15, 16 and 17, where we looked at the ratio of the mean fluorescence intensities. We observed a significant effect between DC/NK and DC/NK/VLP for all 3 surface maturation markers. A WT baculovirus was used as a negative control, in order to see if the significant effect was due to baculovirus/insect cell contaminants. As represented in the figures, we observed that the WT baculovirus showed the same results as NK cells cultured alone or in the presence of DC. So, the significant effect was not due to baculovirus/insect cell contaminants. We now showed that NK cells activated by DC in the presence of VLP, directly induce DC maturation but don't enhance the effect of microbial maturation stimuli. So, we can say that during an infection, before antigen-specific T cells are expanded, NK cells may become activated and amplify the DC maturation induced by VLP, facilitating the activation and expansion of antigen-specific naïve T cells.

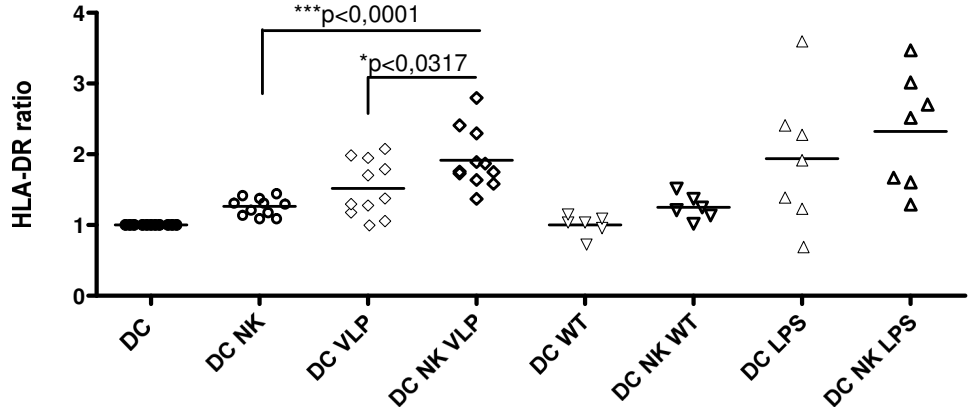

Figure 15: The effect of NK cells and VLP on HLA-DR expression on DC. MFI ratio of HLA-DR to MFI on DC by FACS.

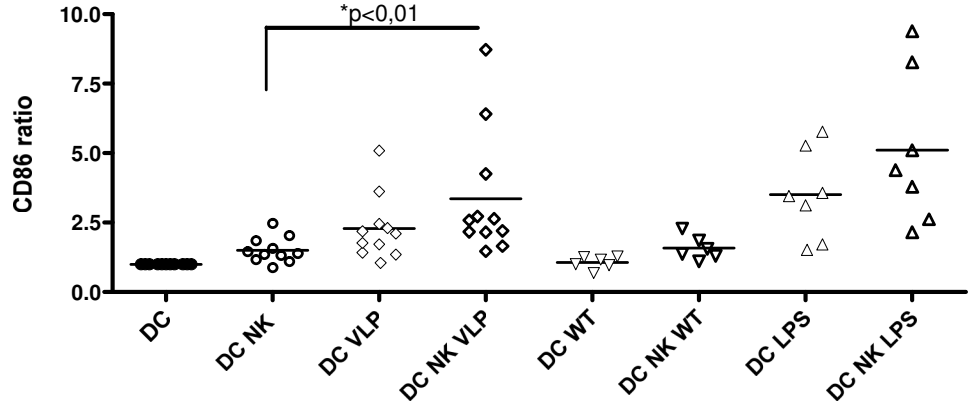

Figure 16: The effect of NK cells and VLP on CD86 expression present on DC. MFI ratio of CD86 to MFI on DC by FACS.

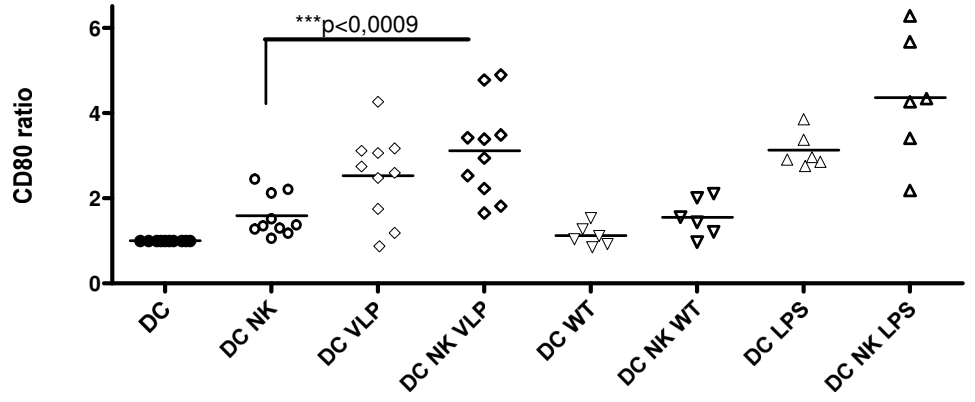

Figure 17: The effect of NK cells and VLP on CD80 expression present on DC. MFI ratio of CD80 to MFI on DC by FACS.

3.7 Production of IL12p70 confirmed that NK cells have a synergistic effect with DC in the presence of VLP

IL-12 is a NK cell stimulatory factor, produced by DC after maturation and is involved in Th1 immune response development. IL-12 is a 75 kDa heterodimeric glycoprotein composed of independently-regulated disulfide-bonded 35 kDa and 40 kDa subunits. The p40 subunit can bind to IL-12 receptors and antagonise the activities of bioactive IL-12. Gerosa et al showed that DC produced IL12p40 after LPS stimulation and that the presence of NK cells in culture with DC increased the IL12p40 production after stimulation with LPS [62]. They also showed that DC stimulated with LPS produce IL12p70, which is the bioactive form, and that the presence of NK cells increased this production. Rudolf et al showed that DC incubated with VLP produced IL12p70. They found low levels of IL12p70 in the supernatants of untreated DC [58].

We confirmed these results (figure 18 and 19) and we studied if NK cells further can stimulate the production of IL12p40 and IL12p70 by DC in the presence of VLP. We did this by performing an IL12p40 and IL12p70 ELISA. The results are shown in figure 18 and 19. For IL12p40 (figure 18), there was a significant effect visible between DC and DC/VLP ($p<0,05$) and between DC and DC/LPS ($p<0,05$). For NK/DC/VLP there was as well an increase of IL12p40 visible in comparison with NK/DC, but this one was not significant. Regarding IL12p70 (figure 19), there was a significant effect visible between NK/DC and NK/DC/VLP ($p<0.05$), between NK/DC and NK/DC/LPS ($p<0.05$), between DC and VLP ($p<0.05$) and between DC and DC/LPS ($p<0.05$). When we performed a paired test, we found as well that there was also a significant effect between NK/DC/VLP and DC/VLP ($p=0,05$). We found that NK cells synergise with VLP in modulating DC cytokine secretion, since the presence of NK cells lead to further increase in IL12p70 production by DC in the presence of VLP. Taken together, these data indicate that NK cells have a synergistic effect with DC in the presence of VLP.

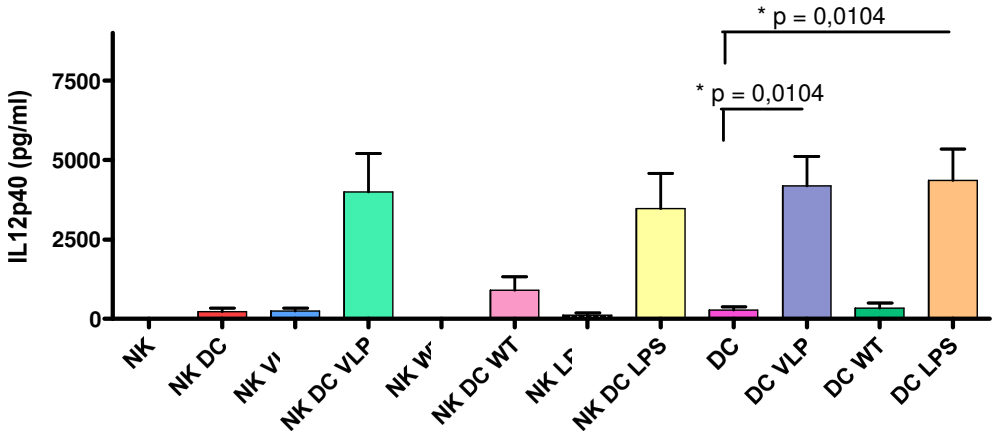

Figure 18: **Production of IL12p40 by DC.**

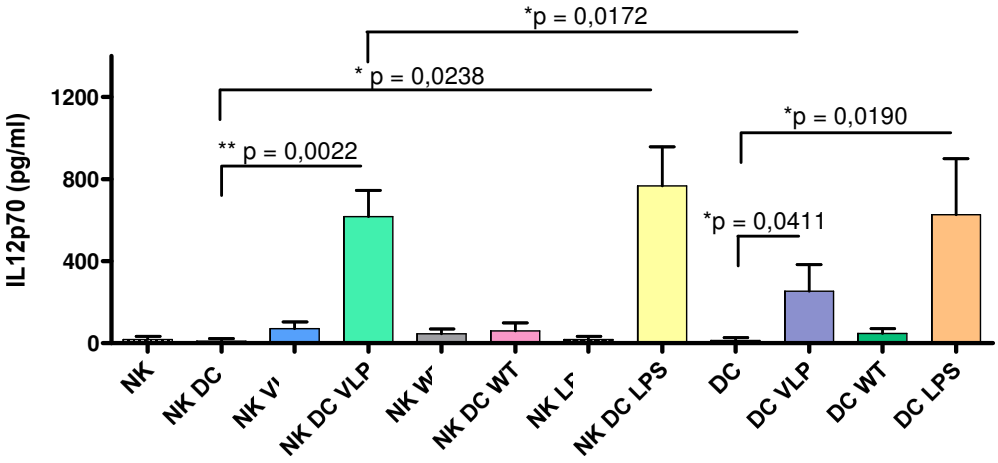

Figure 19: **Production of IL12p70 by DC.**

3.8 IL-10 secretion of human monocyte-derived DC induced by HPV-VLPs

In the literature, it has already been demonstrated that DC activated by LPS can produce IL-10. They showed that LPS can stimulate DC to display similar maturation phenotypes but to differentiate toward an IL-10^{high}- or IL-12^{high}-secretion profile depending on the time they add the maturation signal [63]. Recent studies showed that VLP binds to DC and induce the expression of IL-10. IL-10 is an anti-inflammatory cytokine which inhibits generation of Th1 responses, being implicated in priming Th2 responses. We confirmed this result (figure 20) and we studied if NK cells synergise with VLP in modulating the secretion of IL-10 by DC. We did this by performing an IL-10 ELISA. The results are shown in figure 20. We confirmed that there was a significant effect between DC and DC/VLP ($p<0,0001$). We also saw that there was a significant effect between NK/DC and NK/DC/VLP ($p<0,0001$), but

when we compared the averages of DC/VLP and NK/DC/VLP, we saw that there was no increase present. There was as well a significant effect visible between NK/DC and NK/DC/LPS ($p<0,0005$) and between DC and DC/LPS ($p<0,0001$). When we compared the averages of DC/LPS and NK/DC/LPS we also could observe that there was no increase visible. Therefore we can conclude that no modulation was observed for the anti inflammatory cytokine IL-10.

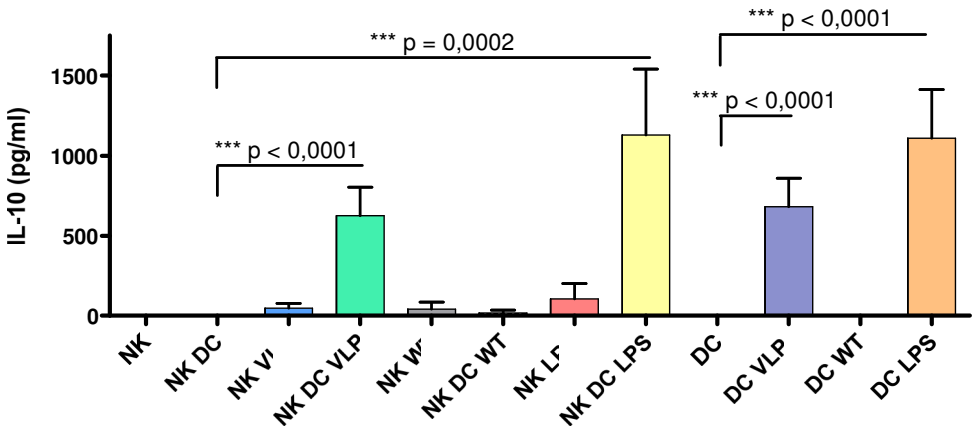

Figure 20: Production of IL-10 by DC.

4. CONCLUSION AND SYNTHESIS

Recently, two prophylactic VLP-based vaccines against HPV have become available on the market. Clinical trails have demonstrated that vaccination with VLP HPV-16/18 L1 leads to a strong humoral immune response. However the clinical trails also showed that these prophylactic vaccines provide limited benefits to women which are already infected with hr-HPV. Literature have already shown that patients with an immune suppression due to HIV infection or organ transplantation have a higher chance to develop cervical cancer [64, 65]. This demonstrates that the immune system plays an important role in cervical cancer. DC and NK cells are components of the innate immune system and recently studies have been focused on the interaction between these two cell types. Fernandez et al., showed that the interaction between DC and NK cells is important to induce an anti-tumour response [22] and Lenz et al., demonstrated that VLP can induce acute activation of DC [59]. So far, NK cell activation in vaccination protocols has been neglected. Because of the importance of a good working immune response to avoid viral immune evasion by HPV, we are interested in the effect of HPV-VLP on DC and NK cells in an autologous setting. Our work hypothesis is that DC activated by HPV-16 VLP could stimulate NK cell responses. Since cross talk between NK/DC are bidirectionally, we will also show the impact of the HPV-VLP on DC in the presence of NK cells.

We observed an increase of the surface expression for CD69 and HLA-DR in the presence of DC and VLP compared with the cocultures NK/DC and NK/VLP. Our results showed that VLP alone is not capable to activate NK cells and thus NK cells become only stimulated in the presence of DC and VLP. The up-regulation of CD69 and HLA-DR is VLP specific since there is no effect observed due to baculovirus/insect cell contaminants. In the literature this cross talk is mediated either by cell-cell contact or to soluble factors. To test this NK cells were incubated with supernatants of DC. These DC were first incubated with VLP, LPS or WT during 24 hours. The preliminary results showed an increase of the surface expression for CD69 but not for HLA-DR. This suggests that the CD69 up-regulation is cytokine-dependent whereas up-regulation of HLA-DR needed cell-cell contact. However, these experiments must be repeated to confirm these observations. A VLP specific ELISA on the DC supernatants needs to be performed as well to determine if there was still VLP present. We also analysed

the effect of HPV-VLP on IFN-γ production, a cytokine which is critical for innate and adaptive immunity against viral and intracellular bacterial infections and for tumour control. We studied if this production can be stimulated via DC activated by VLP. Low amounts of IFN- γ were detected in NK/VLP and NK/DC/WT cultures and the presence of DC and VLP induced a high production of IFN- γ by NK cells (50 fold increase). These results confirmed that NK cells become potent effector cells in the presence of DC and VLP. Interestingly, the results also showed an increased production of IFN- γ by DC in the presence of VLP compared to DC alone. Further investigation of the IFN- γ production by DC could maybe give a new insight into mDC-mediated immune regulation of cytokine network.

Secondly, further activation of DC in the presence of VLP through NK cells was investigated. We observed an up-regulation of HLA-DR, CD86 and CD80 in the presence of NK and VLP compared with NK/DC and NK/DC/VLP. These results show that NK cells can improve the activation of DC in the presence of VLP. This further activation through NK cells was also confirmed by an increase of the IL12p70 production by DC in the presence of VLP. This activation could favour the induction of Th1 response against HPV since the anti-inflammatory cytokine IL-10 was not induced by NK/DC/VLP. There still needs to be determined if the improved activation of DC in the presence of VLP and NK cells was due to a direct effect between NK cells and HPV-VLP or to a indirect effect via the interaction between DC and VLP which stimulates NK cell activation and which will stimulate DC in return. There also needs to be investigated if the interaction is mediated either by cell-cell contact or by soluble factors.
The results of these experiments contribute to a better knowledge about the interaction between DC and NK cells in the presence of the HPV-VLP vaccine and could help to improve the vaccine for the treatment of cervical cancer in humans.

In perspectives, we will study if this NK/DC cross talk really improves the function of NK cells and DC. For NK cells we will do this by studying their cytotoxic activity against HPV positive cell lines and for DC we will study their ability to induce T cell responses.

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